ANALYSIS OF OILSEED GLUCOSINOLATES AND THEIR FATE DURING PRESSING OR DEHULLING

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ABSTRACT

Brassica carinata (A.) Braun and *Camelina sativa* (L.) Crantz are two re-emerging oilseed crops of the Brassicaceae family that are being adapted for cultivation in western Canada. Both seeds of these species reportedly accumulate considerable amounts of sulfur-containing secondary metabolites called glucosinolates. The purpose of the current work was to gain knowledge of the occurrence and distribution of glucosinolates during primary processing of these oilseeds, including during pressing and dehulling. In the first study, a reversed phase HPLC method was developed for the analysis of sinigrin, the major glucosinolate in *B. carinata*. Both C18 columns selected were able to separate the compound with an isocratic eluent containing 100% tetramethylammonium bromide (10 mM, pH 5) delivered at 1 mL/min at a column temperature of 25^{o}C. These chromatographic conditions were applied and sinigrin concentration of whole *B. carinata* seed was estimated to be 29 µg/mg. Average matrix effect was estimated to be 104% that was caused by other components in the *B. carinata* seed matrix. In the second study, high concentrations of glucosinolates were detected and identified in fractions of *C. sativa* seeds using HPLC-ESI-MS. Methods for extraction, isolation, and purification of three individual glucosinolates from these fractions are reported. Quantitation of total glucosinolates was performed on proton NMR using DMF as an internal standard. Quantitation of individual glucosinolates was achieved by using MS extracted ion chromatogram data. Total glucosinolates were found in *C. sativa* whole seed at a concentration of 14 µg/mg, and glucocamelinin, the major glucosinolate, constituted 65% of the total amount. In addition, a dehulling treatment was applied to *C. sativa* seeds, from which both oil content and crude protein content increased after dehulling of the seeds.

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LIST OF ABBREVIATIONS

AITC	Allyl isothiocyanate
APT	Attached proton test
COSY	Correlation spectroscopy
DAD	Diode array detector
DEPT	Distortionless enhancement by polarization transfer
DMF	Dimethylformamide
DL	Detection limit
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
ESI-TOF-MS	Electrospray ionization-time of flight-mass spectrometry
FAB-MS	Fast atom bombardment mass spectrometry
GC	Gas chromatography
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HPLC-ESI-MS	High performance liquid chromatography-electrospray ionization-mass spectrometry
HPLC-MS	High performance liquid chromatography-mass spectrometry
HSCCC	High-speed counter-current chromatography
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MECC	Micellar electrokinetic capillary chromatography
MRM	Multiple reactions monitoring
NMR	Nuclear magnetic resonance
QL	Quantitation limit
RSM	Response surface methodology
TMAB	Tetramethylammonium bromide
UV	Ultraviolet
UV/vis	Ultraviolet visible
RI	Refractive-index

1. INTRODUCTION

Brassica carinata (A.) Braun and *Camelina sativa* (L.) Crantz are two emerging oilseed crops within the Brassicaceae family that are being adapted for cultivation in western Canada (Gugel and Falk, 2006; Rakow and Getinet, 1997). *B. carinata* is commonly known as Ethiopian mustard, and the seeds of *B. carinata* reportedly contain approximately 24.7 to 35.5% oil that is rich in inedible erucic acid (Warwick *et al*., 2006). Improved varieties with higher oil content have been developed (Taylor *et al*., 2010). *C. sativa* is also referred to as false flax or gold-of-pleasure (Budin *et al*., 1995). It is another low-input oilseed species from the Brassicaceae family that exhibited favourable agronomic performance (Gugel and Falk, 2006). The oil content of *C. sativa* seeds is reported to vary from 29.9 to 38.3% (Budin *et al*., 1995), though higher levels (up to 45.2%) have been reported in cultivars selected for oil production (Gugel and Falk, 2006).

Due to the unique fatty acid profiles in seed oils of *B. carinata* and *C. sativa*, research have focused on improvement of the oil quality of these two species, while other components in these oilseeds may also have great potential in several agricultural and industrial areas. Oilseeds of both *B. carinata* and *C. sativa* accumulate considerable amounts of glucosinolates, compounds that are considered to have potential applications as pesticides, health-promoting ingredients, and medicine. Glucosinolates are a group of sulfur-containing plant secondary metabolites, of which, more than 120 known structures have been identified in hundreds of species within 16 plant families, and more than 96 unique glucosinolates have been reported in species of the Brassicaceae family (Fahey *et al*., 2001). These compounds are subject to a hydrolysis reaction catalyzed by an endogenous hydrolytic enzyme, known as myrosinase (EC 3.2.1.147, thioglucoside glucohydrolase) in the presence of moisture upon disruption or damage of the glucosinolate-containing tissue; a glucose molecule and an unstable aglucone are produced from the reaction, and the latter immediately decomposes to a variety of breakdown products that contribute to both sensory and biological activities that are observed in the glucosinolate-producing plants (Fahey *et al*., 2001; Grubb and Abel, 2006). In spite of toxicity, studies conducted on glucosinolates and their hydrolysis products also reveal potential health and nutritional benefits: in addition to the natural role of these compounds in plant defense, some of the biological activities may lead to their use in cancer prevention (Holst and Williamson 2004; Hayes *et al*., 2008).

In mature seed of *B. carinata,* the predominant glucosinolate is sinigrin (prop-2-enylglucosinolate) (Bellostas *et al*., 2007). In *C. sativa,* three glucosinolates are found accumulated in the seed: 9-methylsulfinyl-nonylglucosinolate (glucoarabin, GS9), 10-methylsulfinyl-decylglucosinolate (glucocamelinin, GS10), and 11-methylsulfinyl-undecylglucosinolate (GS11) (Berhow *et al*., 2013; Matthäusa and Zubr, 2000). The value of seed meal generated from these two species is determined, in part, by seed glucosinolate concentration.

1.1 Objectives

Since the biological activities of different glucosinolates and their degradation products differ significantly, it is necessary to have a precise and accurate understanding of the glucosinolate profile and concentration within a given plant. Development of bio-refinery processes that fractionate these two species may be guided by reliable, reproducible, and hopefully fast analytical measurements of glucosinolates in different plant fractions. The objective of this project was to investigate the distribution of glucosinolates in the oilseeds *B. carinata* and *C. sativa* during the processing treatments by use of advanced and developed analytical techniques. The project was conducted as follows:

Objective 1: To develop methods for sinigrin extraction, detection and quantitation

Hypothesis: An RP-HPLC method may be developed that achieves retention of sinigrin (k=1) on modified reversed phase silica C18 columns.

Objective 2: To measure sinigrin levels in seed fractions during the processing of the oilseed *B. carinata*

Hypothesis: Plant matrix effects interfere with the measurement of sinigrin, causing underestimation of the sinigrin present.

Objective 3: To develop methods of extraction, isolation, detection and quantitation for *C. sativa* glucosinolates

Hypothesis: Quantitative analysis of the three glucosinolates in fractions of *C. sativa* seeds may be achieved through HPLC-MS or NMR techniques.

Objective 4: To compare the levels of individual glucosinolates in different seed fractions during dehulling and pressing of the oilseed *C. sativa*

Hypothesis: The concentration of individual glucosinolates in *C. sativa* seed fractions varies during oilseed processing; Fractions that have the highest concentration of glucosinolates could be used as the raw material for recovery of glucosinolates.

2. LITERATURE REVIEW

2.1 The mustard family

Canola, flaxseed and soybean are the major Canadian oilseed crops; of these, canola makes up the largest portion (Figure 2-1). The official definition of canola is as follows:

> Seeds of the genus *Brassica* (*Brassica napus*, *Brassica rapa* or *Brassica juncea*) from which the oil shall contain less than 2% erucic acid in its fatty acid profile and the solid component shall contain less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free solid (Canola Council of Canada, 2013).

The first canola was developed in the 1970s in Canada from rapeseed (Canola Council of Canada, 2013) though subsequently *Brassica juncea* mustard varieties have been developed that are also called canola. Rapeseed, mustard and canola, along with many other species that are of agricultural and economical importance, including broccoli, cabbage, radish, cauliflower and turnip, all belong to the plant family Brassicaceae (Callihan *et al*., 2000; Hayward, 2012). Also referred to as Cruciferae, the Brassicaceae family consists of more than 3,700 species in 338 genera, all of which share three anatomic characteristics, including tetradynamous stamens, cross-shaped flowers, and replum of the seedpods (Callihan *et al*., 2000; Hayward, 2012).

With increased oilseed demand in Canada and worldwide, the need for exploration of alternate oilseeds as rotation crops or for expanded growth in harsh environments becomes clear. *Brassica carinata* and *Camelina sativa* are two ancient oilseed crops within the Brassicaceae family that have the potential for expanded cultivation in Canada. The values of these two crops will increase if more advantageous properties are revealed from fractions of the plants. As one of many examples, the three-year "Prairie Gold" project, which was funded through the Western Economic Partnership Agreement (WEPA) in Canada, was initiated for optimization of the cultivars of *B. carinata* and *C. sativa* to produce oils for biofuel and non-biofuel uses, such as lubricants, polymers, and jet fuel by application of genomic techniques (Prairie gold, 2013).

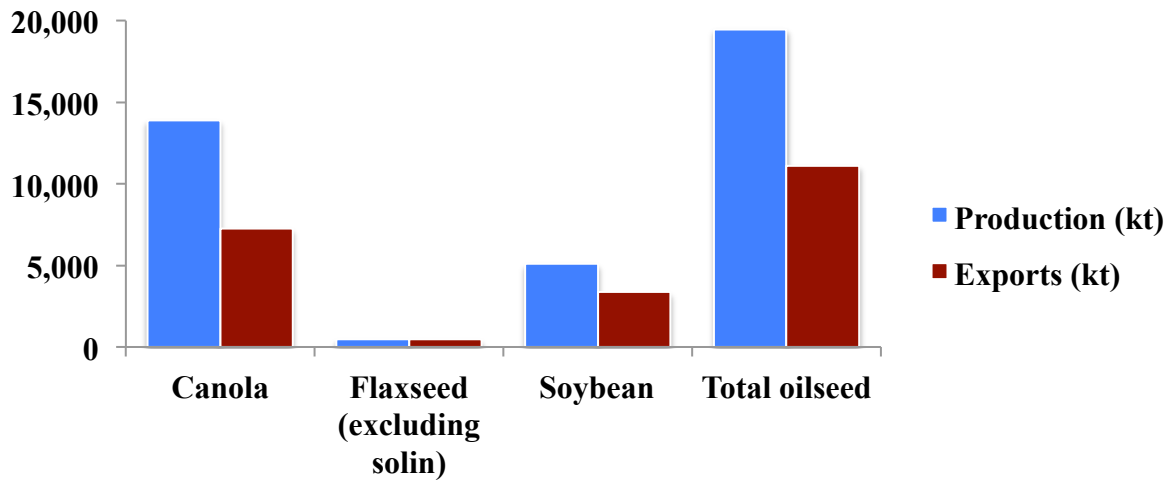


Figure 2-1 Oilseed crops production and exports in Canada in the 2012-2013 crop year (modified from Agriculture and Agri-Food Canada, 2013).

2.1.1 *Brassica carinata*

The genus *Brassica* within the Brassicaceae family has a collection of species with considerable genetic diversity and agriculture uses: it contains over 30 species including broccoli, cauliflower, Brussels sprouts and cabbage, many of which are consumable (Hayward, 2011). The "Triangle of U" that was first published in 1935 illustrates the relationship between three major ancestral diploid *Brassica* species, which are *B. nigra* (black mustard), *B. oleracea* and *B. rapa*, as well as their amphidiploid hybrids, *B. carinata* (Ethiopian mustard), *B. juncea* (brown mustard) and *B. napus* (rapeseed), each of which contains four genomes from both parents (Hayward, 2011; Nagaharu, 1935; Østergaard and King, 2008; Snowdon *et al*., 2007).

B. carinata (A.) Braun is an amphidiploid that arose as the result of the hybridization between *B. nigra* and *B. oleracea*. Traditionally known as Ethiopian mustard or Abyssinian mustard, it is an oilseed crop that is being adapted for cultivation in western Canada (Snowdon *et al*., 2007). The agronomic adaptability of *B. carinata* in the dry prairies of western Canada as an oilseed crop has been evaluated since two decades ago (Getinet *et al*., 1996; Rakow and Getinet, 1997). Notable agronomic advantages of this plant include resistance to heat, drought and insect diseases, all of which contribute to its potential as an alternate oilseed crop or rotation crop with canola (Rakow and Getinet, 1997). The seeds of *B. carinata* are similar in size and shape to canola (Figure 2-2). Oil content of *B. carinata* seeds is reported to range between 24.7 to 35.5%,

while some accessions that have been selected for increased oil content cultivation contain over 40% oil (Warwick *et al*., 2006). The seed oil typically contains high concentrations of unsaturated fatty acid, with erucic acid taking up the largest portion (Table 2-1), which, along with its derivatives, has wide industrial uses including as precursors for production of plastic, resins, cosmetics, and surfactants (Taylor *et al*., 2010; Warwick *et al*., 2006). Through traditional breeding and genetic modification, *B. carinata* lines with higher oil content and/or long-chain fatty acids could be realized (Taylor *et al*., 2010).

At the moment, cultivar improvement of *B. carinata* is largely focused on optimization of its seed oil for biofuel production and industrial uses. In Canada, *B. carinata* has not been designated as an official grain, which means that this species is not regulated under the Canada Grain Act (Canadian Grain Commission, 2014). This species is mostly cultivated under contract for business development or projects, such as the "Prairie Gold" project. Promising results were obtained that motivated commercial interest in the production of this plant. For example, Agrisoma Biosciences Inc. is a biotechnology company that aims to improve *B. carinata* for use in biodiesel and biojet fuel by means of the company's patented technology called Engineered Trait Loci (Agrisoma, 2013b). In a press release, Agrisoma reported that trademarked varieties of Resonance® *B. carinata* were planted and harvested on 50 acres in 2011 and 6000 acres in 2012 (Agrisoma, 2013a).

Figure 2-2 Seed coats and cotyledon of a plot of *B. carinata* seeds. (Provided by Dr. Kevin Falk of Agriculture and Agri-Food Canada, Saskatoon, SK, Canada).

Table 2-1 Fatty acid profiles and concentrations (%) of 66 accessions of *B. carinata* (Warwick *et al*., 2006)

	Mean	SD	Range
C18: 1 – Oleic	7.7	1.1	5.1–11.6
C18: 2 – Linoleic	16.1	0.9	13.7–18.9
C18: 3 – Linolenic	13.3	1.1	10.2–16.0
C20: 1 – Eicosenoic	7.6	0.8	6.2–12.0
C20: 2 – Eicosadienoic	1.1	0.1	1.0–1.3
C22: 1 – Erucic	42.1	2.2	30.9–45.7
C22: 2 – Docosadienoic	1.7	0.2	1.0–2.4
C24: 1 – Nervonic	2.5	0.2	2.1–3.4
Other fatty acids	1.6	0.2	0.8–2.2
Total saturated fatty acids[a]	6.2	0.3	5.7–8.0

[a] C14: 0 (myristic), C16: 0 (palmitic), C18: 0 (stearic), C20: 0 (arachidic), C22: 0 (behenic) and C24: 0 (lignoceric)

Table 2-2 Fatty acid profiles and concentrations (%) of 13 accessions of *C. sativa* (Budin *et al*., 1995)

	Trial 1	Trial 2	Overall mean
C16: 0 – Palmitic	7.0	6.0	6.4
C18: 0 – Stearic	2.5	3.1	2.8
C18: 1 – Oleic	15.4	16.3	15.9
C18: 2 – Linoleic	21.5	20.5	20.9
C18: 3 – Linolenic	33.2	29.2	30.7
C20: 1 – 11- Eicosenoic	12.6	14.2	13.6
C22: 1 – Erucic	2.2	3.6	3.0
Others	5.6	7.2	6.6

2.1.2 *Camelina sativa*

Camelina sativa (L.) Crantz, also known as false flax or gold-of-pleasure, is another oilseed crop of the Brassicaceae that is being improved for increased cultivation in western Canada (Gugel and Falk, 2006). Agronomic performance and seed quality of this species have been investigated earlier (Gugel and Falk, 2006; Vollmann *et al*., 2007). The oil content of *C. sativa* seed ranges from 29.9 to 38.3% (Budin *et al*., 1995), and 38 to 43% (Gugel and Falk, 2006). The oil typically contains high concentrations of unsaturated fatty acids (saturated/unsaturated ratio 0.11), tocopherol (γ-tocopherol 78 mg/100g), plant sterol (511 mg/100 g), and cholesterol (35 mg/100g) (Budin *et al*., 1995; Schwartz *et al*., 2008). This unique fatty acid composition (Table 2-2) gives *C. sativa* oil great potential to be applied in many areas such as fish oil replacement, jet fuel, biodiesel feedstock, foods and dietary supplements (Hixson *et al*., 2013; Small, 2013).

One of the most notable areas of research being conducted on the oil extracted from this species has been the assessment of the *C. sativa*-based biofuel. In 2011, the US air force made a successful test flight with an aircraft powered by 50/50 *C. sativa*-based biofuel; their plan is to apply it to 50% biofuel by 2016 (http://www.standard.net/stories/2011/07/29/air-force-wants-50-use-biofuel-2016). Additionally, the high level of essential fatty acids (linolenic 30.7%; linoleic 20.9%) makes the oil potentially beneficial for human nutrition (Budin *et al*., 1995). In January 2010, Health Canada notified a food manufacturing company that it "takes no objection to the use of cold-pressed *C. sativa* oil as a food ingredient" (Health Canada, 2013). This is interesting as *C. sativa* oil contains an erucic acid level that is above that allowed in canola.

Seed meal of *C. sativa* recovered from oil extraction is a by-product that contains considerable amounts of residual oil, protein, carbohydrates and other phytochemicals (Berhow *et al*., 2013; Matthäus and Zubr, 2000). Effective utilization of this by-product could potentially add to its economic value. However, the seed meal has not yet been allowed as animal feed according to Health Canada. In Europe, *C. sativa* is listed as undesirable substances in animal feed, and no "quantitatively determinable amount" is allowed to be present in feedstuffs (EC Directive, 2002). There is evidence that *C. sativa* seed meal used as feed (5 and 10%) to broiler chickens resulted in decreased growth and feed intake, while it increased meat omega-3 fatty acid levels (Ryhänen *et al*., 2007). *C. sativa* seed meal also contains glucosinolates, sinapine, condensed tannins, phytic acid, inositol phosphates and heavy metals. The presence of these

compounds may influence the utility of *C. sativa* meal in meeting animal nutrition needs (Matthäus and Zubr, 2000).

2.2 Primary processing of oilseeds

Primary processing of oilseed by pressing may involve up to three operations: a pre-treatment step (conditioning), the main oil-pressing step, and a following optional solvent extraction step that is aimed to recover oil from seed cake or seed meal (Mulder *et al*., 2012). Due to the novelty of *B. carinata* and *C. sativa*, processing information for extracting the seeds is scarce. Comparatively, more information of the processing of the oilseed canola and/or rapeseed is available. Due to the close relationship between these crops (all in the Brassicaceae family), including the similar appearance and composition, the processing of canola and/or rapeseed is described in literature as an example of the primary processing of these oilseeds (Figure 2-3).

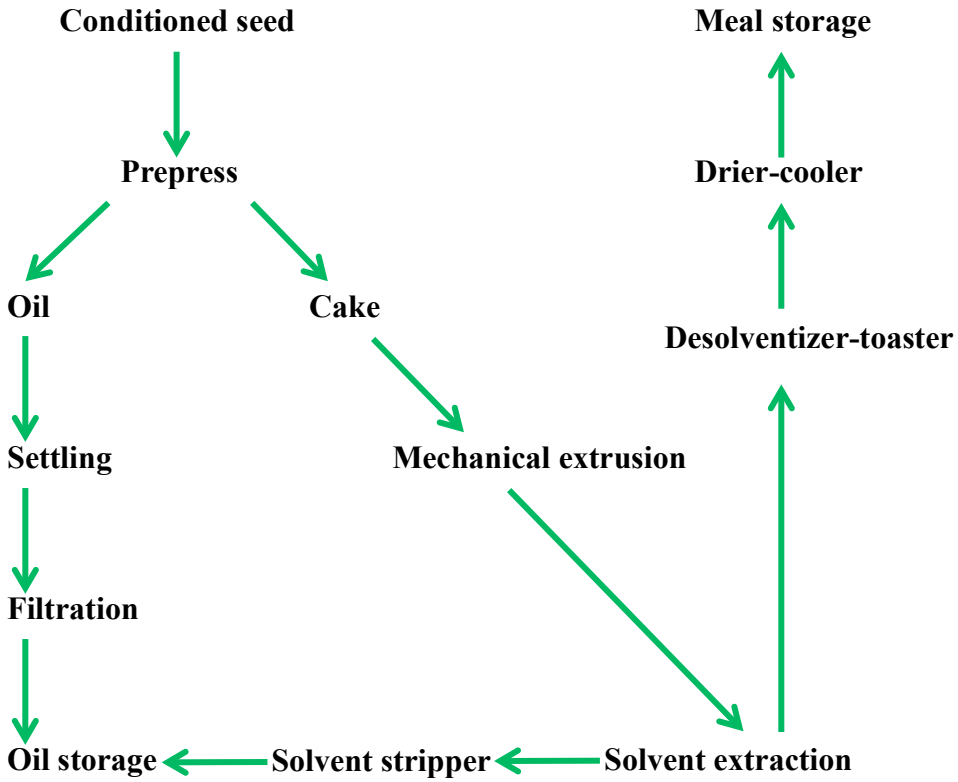


Figure 2-3 *Brassica* seed processing (modified from Carr, 1995).

2.2.1 Pre-treatments

Pre-treatments of oilseed that prepares them for oil extraction may include dehulling, thermal pre-treatment, enzyme treatment, application of pulsed electric field, etc., from which seeds are conditioned before oil extraction (Mulder *et al*., 2012).

Dehulling, though rarely practiced in canola, is potentially an important step in the pre-treatment of oilseed. Seed hull contains a high percentage of fiber with poor digestibility (Bell, 1995). Removal of seed hull is expected to improve the value of both oil and seed meal. Oil from dehulled seed may have improved color, while seed meal will have a decreased amount of fiber, and an increased amount of protein. Dehulled meal will have improved nutritional value in animal feed (Ikebudu *et al*., 2000). Mulder *et al*. (2012) summarized five kinds of dehulling equipment or techniques for rapeseed or canola that have been recognized or proposed, which included the use of CETIOM dehullers, abrasive dehullers, rolls dehullers, combined treatment of infrared and microwaves, and tail-end hull separation. The CETIOM dehuller is a patented device developed in the 1970s that was devised to separate seed hull and meat by centrifugal forces: after shattering the seed a portion of the hull 12-13% was separated from the rapeseed embryo using aspirators (Mulder *et al*., 2012). Abrasive dehullers remove the seed hull by placing seed on a rotating disc; the grit size of the rotating disc may be selected for different seed characteristics (Ikebudu *et al*. 2000). A rolls dehuller is a device that has two rolls separated by a gap that is narrower than most seed; the seed is fed between the rolls and splits if the conditions are optimized (Mulder *et al*., 2012). Treatments, such as infrared and microwaves can be applied to seeds in order to create more space between the gap of seed hull and meat: following these conditioning treatments mild technique such as impact or passing through gapped rolls may be more effective at dehulling (Mulder *et al*., 2012). Tail-end hull separation takes place after oil extraction, where seed meal is milled before being separated from seed hull by air (Mulder *et al*., 2012). In Canada, canola dehulling is not practiced and this is due to technical issues encountered during operation, including small seed size, the close gap between seed hull and cotyledon that have made dehulling inefficient. The loss of oil to the hull fraction during dehulling can make the process uneconomical (Canola Council of Canada, 2014; Ikebudu *et al*., 2000). Mulder *et al*. (2012) also addressed the technical difficulties in crushing the softer kernel after dehulling: since the canola hull provides resistance during pressing, presses that are designed for high fiber seeds do not readily remove oil from the seed. Another challenge may

take place, where the heat used in the removal of hexane would compromise protein solubility. However, it has been demonstrated that removing hexane from hull requires more heat than removing hexane from dehulled oilseed meal. By using a certain kind of pre-treatment, such as hydrating followed by heating the canola seeds, the efficiency of dehulling was shown to improve (Ikebudu *et al*., 2000).

Thermal treatment is another common method for rapeseed pre-treatment processing. Data has shown that cooking the seeds helps to destabilize the oleosins and decrease the moisture content of the seed, which assists in further oil pressing, while it may also decrease protein solubility (Mulder *et al*., 2012). Thermal treatment may also help to inactivate enzymes, especially myrosinase (Carr, 1995).

2.2.2 Oil pressing

Mechanically prepressing the oilseed prior to performing solvent extraction of the seed cake is generally considered an efficient practice for seed with high oil content (> 20%) (Carr, 1995).

During pressing, some solids are released into the oil due to the pressure and shear in the expeller. Crude oil contains a wide range of these solids. Screening and settling of larger solids typically occurs in a screening tank while filtration removes smaller particles. The settled and filtered solids are called “foots”, and can be returned to the expeller for re-pressing or pressed in a special “foots” screw press (Carr, 1995). Oil may be recovered from foots through screening or centrifugation as well.

2.2.3 Solvent extraction

The purpose of the solvent extraction step in oilseed processing is to recover oil from the seed cake or other solid materials after oil pressing. The solvent works to dissolve and separate the oil from the seed cake (Carr, 1995). Hexane is approved for this application and the only solvent used by most manufacturers. Other organic solvents including isopropanol, ethanol and methylene chloride have also been suggested for oil recovery (Johnson and Lusas, 1983). Enzyme-based aqueous extraction (Rosenthal *et al*., 1996) and supercritical fluid (SC-CO_2) extraction (Temelli, 2009) have also been suggested as replacements for hexane due to safety and health concerns. Seed meal from solvent extraction contains 30-35% solvent, which is removed by a desolventizer-toaster. After this step, the seed meal - which contains

approximately 1% lipids and over 15% moisture - is sent to a drier-cooler before storage (Figure 2-3) (Carr, 1995).

2.2.4 Oil processing

The combined crude oil from the pre-pressing and solvent extraction steps contains a small amount of impurities such as gums (phospholipids), free fatty acids, waxes, and pigments. The crude oil is then put through another processing chain to remove these impurities and the general scheme includes degumming and/or alkali refining, physical refining (for free fatty acid and odor), bleaching (for pigments), and winterization (for waxes) (Carr, 1995).

2.3 Glucosinolates

2.3.1 General aspects of glucosinolates

Glucosinolates are a group of sulfur-containing plant secondary metabolites present commonly in many species belonging to the family Brassicaceae (Fahey *et al*., 2001). Numerous researches have done on several aspects of this class of compounds. Grubb and Abel (2006) extensively reviewed the biosynthesis of glucosinolates and the compounds' regulation at a molecular level while Fahey *et al*. (2001) reviewed the occurrence, chemical classification and distribution of glucosinolates and their decomposition products among plants. According to Fahey *et al*. (2001) at least 120 different glucosinolates have been identified in 16 families of the order Capparales, and the Brassicaceae family alone contains at least 96 of them. A more recent review by Clarke (2010) documented the discovery of new glucosinolates where up to 200 unique glucosinolates were cited. Despite this diversity, compounds in the group all share the same chemical skeleton (Figure 2-4): every glucosinolate is characterized by a central carbon atom that is attached to a glucose ether group via a sulfur atom, to a sulfate group via a nitrogen atom, and to a side chain (R) that is derived from different amino acids, which further classify the compounds to be aliphatic, benzenic, and indolic (Agerbirk and Olsen, 2012).

While glucosinolates are relatively stable in their intact form, they are susceptible to hydrolysis by the enzyme myrosinase (E.C. 3.2.1.147), a β-thioglucohydrolase that is stored in myrosin cells in the plant, a location that is sequestered from contact with glucosinolates (Bones and Rossiter, 2006; Fahey *et al*., 2001). The hydrolysis reaction takes place upon injury (wounding, mastication, freezing or grazing) of plant tissues, where the glucosinolate- and myrosinase-containing tissues are damaged; the loss of cell integrity with concomitant mixing of

the glucosinolate and the enzyme myrosinase enables enzymatic hydrolysis (Figure 2-5) where the cleavage of the thioglycoside bond leads to the release of a glucose molecule and an unstable aglucone, and the latter compound decomposes either spontaneously or it is subject to the action of a myrosinase modifier protein, epithiospecifier protein (Grubb and Abel, 2006). After hydrolysis of the thioglucoside, a variety of breakdown products are produced that contribute to both sensory properties and biological activities observed in glucosinolate-containing plants (Fahey *et al*., 2001; Grubb and Abel, 2006; Holst and Williamson, 2004). The kinds of products of glucosinolate-myrosinase action depend on both the chemical structures of the parent glucosinolates and the conditions of the reaction, such as pH, the presence of metal ions (Fe^{2+}) and epithiospecifier protein, and temperature (Bennett *et al*., 2004; Fahey *et al*., 2001; Grubb and Abel, 2006; Rask, *et al*., 2000).

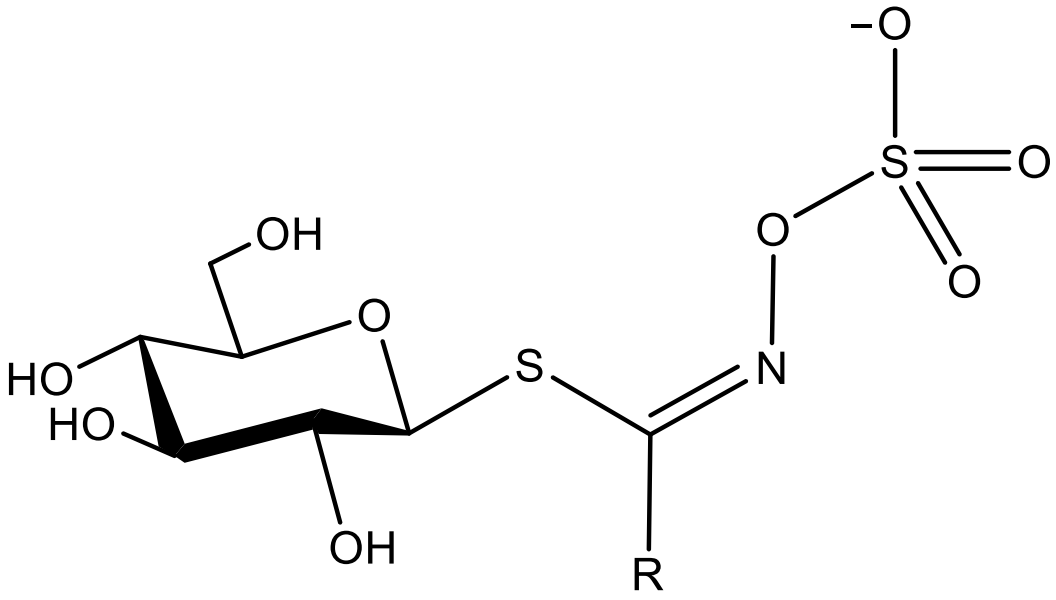


Figure 2-4 Basic glucosinolate skeleton.

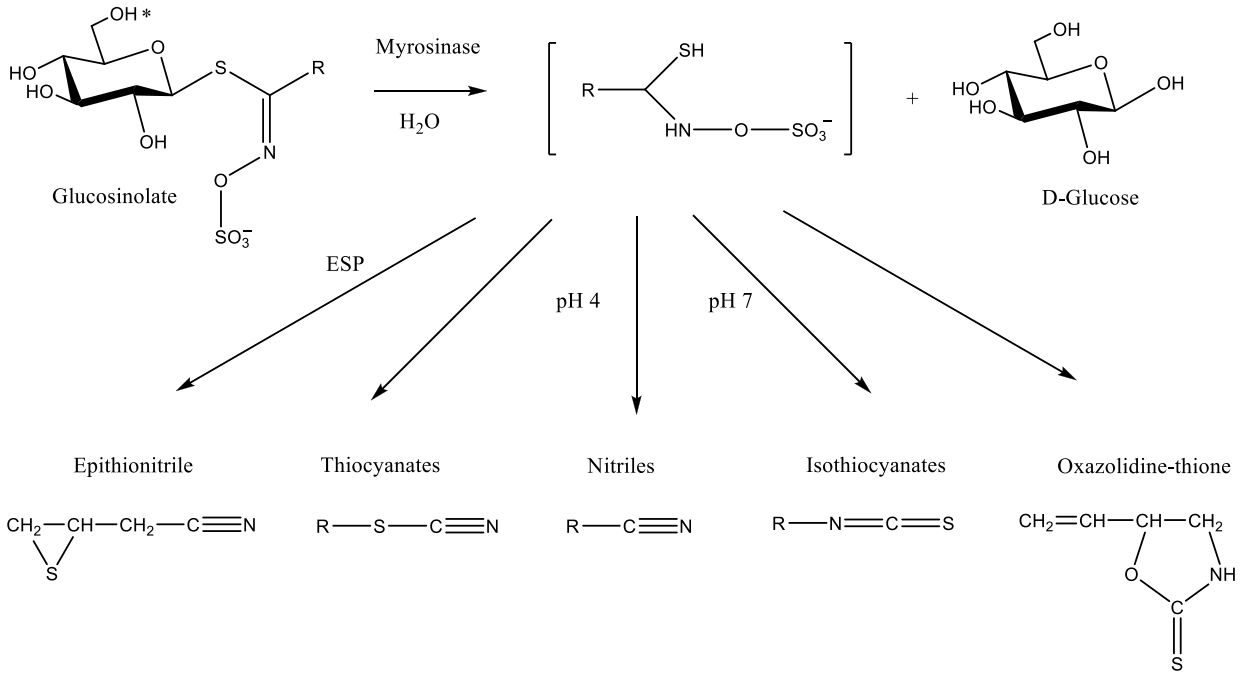


Figure 2-5 Enzymatic hydrolysis of glucosinolates (modified from Rask *et al*., 2000).

Traditionally, glucosinolates were considered undesired in plants for animal feed due to the deleterious impact of their hydrolysis products on animal performance, which is often measured by growth. The most recognizable of these undesirable products include isothiocyanates, thiocyanates, nitriles and oxazolidinethiones (Alexander *et al*., 2008). The physiological and possible toxic effect these compounds may have on animal growth when glucosinolates were consumed in feed can be found in an earlier review written by Fenwick and Heaney (1983). More recent evidence shows that isothiocyanates may be mutagenic and carcinogenic; thiocyanates and oxazolidinethiones are able to impact the thyroid function, which subsequently leads to damage in growth and reproduction; and the formation of nitriles causes damage in liver and kidney functions (Alexander *et al*., 2008; Holst and Williamson, 2004). In contrast to these studies, more research has been focused on the chemopreventive and health effect of these compounds on animals and human. One example has been the glucosinolate glucoraphanin, which decomposes to sulforaphane upon hydrolysis: this product has been shown to have cancer chemopreventive and inhibitory activity (Fahey and Talalay, 1999; Fahey *et al*., 2003). Holst and Williamson (2004) extensively reviewed the bioavailability of glucosinolates and their hydrolysis products, as well as areas of future research while Hayes *et al*. (2008) critically reviewed the formation of common glucosinolate hydrolysis products and the cancer

chemopreventive functions of these products. As implied by the authors, many of the compounds' biological properties and the mechanism of their interrelation with mammalian cells remain unknown (Hayes *et al*., 2008). Therefore, the accurate identification and quantitation of glucosinolates from various plant sources, and their fate during the processing of these sources are necessary.

2.3.2 Extraction and isolation of glucosinolates from plant tissues

Numerous works have been performed on the analysis of individual glucosinolates present in cruciferous plant materials. Successful determination of glucosinolate content in plant material consists of two major components; the first is the complete and quick extraction of the compounds from plant (biological) materials.

A major objective of sample preparation for chromatographic or spectroscopic analysis is the predictable isolation and enrichment of analytes from extracts (Oliveira *et al*., 2007). A primary extract is often obtained from an extraction step prior to isolation and enrichment, and the purpose is to uniformly recover analytes present in the matrix (Seidel, 2012). While methods such as high temperature/pressure treatment and acid/alkali digestion are often applied for preparing inorganic analytes from extracts, organic compounds in different matrices are instead extracted by means of phase extraction, which can involve polymers, organic solvents and porous solids (Oliveira *et al*., 2007).

Extraction efficiency is influenced by temperature, the extraction solvent, solvent-to-material ratio, agitation, pressure and extraction time. Heat is a common treatment for increasing extraction efficiency before and/or during extraction of glucosinolates. Many published methods described extraction at temperatures between 70 and 100^{o}C in order to inactivate the endogenous myrosinase (Cools and Terry, 2012; ISO, 1992; Kiddle *et al*., 2001; Szmigielska and Schoenau, 2000; Wathelet *et al*., 1999). Nevertheless heat treatment is controversial. Tsao *et al*. (2002) pointed out that heating before extraction may not inactivate myrosinase completely, especially when the moisture content is less than 8%. In other cases, heating may lead to degradation of glycosides like glucosinolates. Additional discussions regarding more severe thermal degradation of glucosinolates are reviewed in section 2.4.2.

Another way of inactivating myrosinase is to perform microwave treatment. It has been shown that microwave pre-treatment of the plant material not only helps inactivate the enzyme, but also increases the extractability of many glucosinolates, with the behavior of individual

glucosinolates varying under different energy inputs of microwave (Oerlemans *et al*., 2006; Verkerk and Dekker, 2004).

Extraction protocols for glucosinolates vary significantly between analytical procedures regardless of the detection method used. The European Community (EC, 1990) and the International Organization for Standardization (ISO, 1992) adopted the RP-HPLC analysis of desulfoglucosinolates as the official reference method for the determination of individual glucosinolates. This method, which involves aqueous methanol extraction, purification and enzymatic desulfation by sulfatase prior to HPLC analysis, is still in use in many laboratories (Agerbirk and Olsen, 2012; Hennig *et al*., 2012; ISO, 1992). In 1999, Wathelet and co-workers (1999) modified this ISO protocol: specifically for extraction, single extraction (instead of twice in the ISO protocol) using aqueous methanol (70% (v/v); 75^{o}C) was proposed. As demonstrated in the ISO protocol, a standard desulfation reaction takes approximately 16 h or overnight, where purified sulfatase (usually sulfatase (EC 3.1.6.1) from *Helix pomatia)* is added to a pre-equilibrated DEAE anion exchange column following the loading of centrifuged supernatant from extracts (ISO, 1992), but the protocol of desulfation in practice varied in different publications (Kiddle *et al*., 2001; Oerlemans *et al*., 2006; Wathelet *et al*., 1999). Hennig *et al*. (2012) attempted to demonstrate an appropriate desulfation procedure for desulfoglucosinolates determination in microtiter plates, and they reported that the concentration of sulfatase applied should be restrained within a narrow range to avoid overestimation or insufficient desulfation. In addition, evidence has been shown that desulfation is prone to induce further transformation of desulfoglucosinolates (De Nicola, 2013).

In addition, drying and grinding are approaches that are conducted to help provide a more stable and homogenous sample and to improve the efficiency of plant metabolite extraction. The drying step assists in protection of metabolites of interest from degradation while grinding the materials increases the surface area, which facilitates solvent penetration (Seidel, 2012). Common drying methods for glucosinolate-containing plants include freeze-drying, oven-drying and liquid nitrogen (Oerlemans *et al*., 2006). Kiddle *et al*. (2001) recommended the use of liquid nitrogen to flash-freeze fresh materials and a storage temperature below -20^{o}C.

Natural products are mostly extracted from plant solids by solvent extraction. Polar solvent is effective for extracting polar compounds like glucosinolates. The use of organic solvent can also achieve the denaturation of endogenous enzyme in plant samples without

applying heat. In many reported cases, aqueous methanol (polarity index 5.1) solution has been used. Aqueous methanol (70% (v/v); 75^{o}C) has been reported in the ISO 9167-1 method (1992) described above. On the other hand, some doubts regarding the use of methanol have been proposed, suggesting that the solvent could react with isothiocyanates (Tsao *et al*, 2002 and reference herein). Tsao *et al*. (2002) compared five techniques for extracting sinigrin from mustard (*B. juncea*) materials, and results showed that using boiling 50% (v/v) aq. acetonitrile followed by reflux was generally the most efficient approach for recovering glucosinolates from both bran and seed. Cools and Terry (2012) simplified this method and compared it with three other extraction methods for sinigrin from mustard (*B. juncea*) seed for later HPLC analysis, and concluded that boiling 50% (v/v) aq. acetonitrile again was more efficient than other methods for sinigrin. Other solvents such as aqueous ethanol, boiling water, phosphate buffer, phosphoric acid, and methanol-water-ammonium solution have also been investigated for extracting glucosinolates as summarized and detailed by Clarke (2010), while the author recommended the 70% methanol as the default solvent. Heavy water was used as the solvent to extract sinigrin from Oriental mustard (*B. juncea*) meal for later proton NMR analysis (Belliveau and Romero-Zerón, 2010).

Isolation of natural products consists of extraction and purification, and is often conducted on plant materials with high concentrations of the target compounds. Reproductive tissues, and especially seed, have been reported to contain the highest concentration of glucosinolates. This is consistent with the compounds' natural role in defense (Bennett *et al*., 2003; Clarke, 2010; Fahey *et al*., 2001). Seed is also an ideal source for glucosinolate isolation. In the meticulous work by Bennett *et al*. (2004), cruciferous seed and some related wild and weed were screened and identified as sources for isolation and purification of specific glucosinolates. For isolation of glucosinolates from plant materials, extracts prepared as described above could be isolated using ion-exchange columns, flash chromatography, high-speed counter-current chromatography (HSCCC), and preparative HPLC (Barillari *et al*., 2005; Fahey *et al*., 2003; Kpeterka and Fenwick, 1988; Rochfort *et al*., 2006). In a more recent publication, Berhow and co-workers (2013) demonstrated a protocol to isolate glucosinolates from defatted seed meal of *C. sativa*, using a selective combination of reversed phase flash chromatography, preparative HPLC, counter-current chromatography, and ion exchange chromatography; it was also noted that the use of counter current chromatography prior to

preparative HPLC resulted in higher yields (Berhow *et al*., 2013).

2.3.3 Detection of glucosinolates

Detection of glucosinolates has been traditionally achieved by the identification of their enzymatic degradation products using methods based on the determination of glucose or thiocyanate ion released from hydrolysis, and therefore the compounds were originally determined as a total group (Olsen and Sørensen, 1981).

Modern spectroscopic methods that allow detection of individual glucosinolates have been developed and the ones being mostly used are chromatographic separation coupled to diode array detector (DAD) and/or mass spectrometry (MS) detector. Mass spectrometry is a technique that has proven excellent in the detection of plant metabolites with high sensitivity and selectivity over other types of detection techniques, and it is capable of providing additional details regarding the structure of analytes. Mass spectrometric analysis of intact glucosinolates has been reported using fast atom bombardment (FAB) mass spectrometry under both positive and negative modes (Self, 1987), negative electrospray-quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) (Cai *et al*., 2004), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Botting *et al*., 2002).

Among the analytical detection techniques available, nuclear magnetic resonance (NMR) spectrometry has been embraced for its ability to unequivocally characterize and/or confirm structures of dissolved organic compounds. This method is especially useful for measuring the resonances of proton (^{1}H NMR) and/or carbon (^{13}C NMR) signals in a spectrum that can be used to elucidate connectivity and configuration of compounds. Combined methods of analysis are best when studying complex compounds or mixtures. For example, when compounds with similar structures happen to have the same nominal ion mass and fragmentation behavior by mass spectrometer, NMR spectrometry makes distinction between these compounds possible. This is beneficial for the analysis of glucosinolate isomers given their consistent general structures. In a review on glucosinolate structures, Agerbirk and Olsen (2012) specifically emphasized the importance of NMR analysis for structural elucidation of both known and novel glucosinolates and their breakdown products. In a series of analyses for glucosinolates from plant extracts, Prestera *et al.* (1996) identified 14 intact glucosinolate standards, in the form of standards and/or paired-ion chromatography fractions, and developed a proton NMR database for these glucosinolates, which detailed the chemical shifts of R groups and glucosinolate functional

groups. Belliveau and Romero-Zerón (2010) reported a protocol for extracting the glucosinolate sinigrin from *B. juncea* meal, in which ^{1}H NMR spectroscopy was used to identify the compound and monitoring its degradation by myrosinase (Belliveau and Romero-Zerón, 2010). While this experiment was performed on a qualitative level, it proved the potential and advantages of quantitative NMR in measuring glucosinolates in *Brassica* seed fractions.

2.3.3.1 High performance liquid chromatography detection of glucosinolates

The first commercial high performance liquid chromatography (HPLC) instrument was introduced in 1968. The technique was called high-pressure liquid chromatography at the time (Ettre, 2005). Ever since then, the application of this technique has benefited many research areas in life sciences. A typical HPLC system includes a sample injector, a pump, and a detector. Depending on the nature of analytes in the mixture, a column with specific bonding material is connected to the system. The sample injector places the sample into the flow of mobile phase upstream of the column. The mobile phase is pumped to the column under high pressure. When eluting compounds reach the detector, signals are produced as a function of the amount of analyte.

HPLC separation can serve in isolation and purification of analytes and in large-scale production of pure compounds. The method is suitable for the separation of bioactive biopolymers, as well as in the monitoring and quantitation of specific compounds in complex biological materials (Unger *et al*., 2010). The mobile (solvent) and the stationary phase (column) are used in combinations to affect a wide range of separations. The mechanism of separation is based on the distribution of compounds in the sample on the stationary phase and the mobile phase (Unger *et al*., 2013). Extensive mathematical models are available to describe the efficiency and capacity of chromatographic processes. The van Deemter plate-height equation (Equation 2.1) belongs to the macroscopic lumped-kinetic model, and it relates the column performance over a range of flow and kinetic parameters (Feliner and Cavazzini, 2013), in which height equivalent of a theoretical plate was a mathematical concept proposed to characterize the column efficiency. A more direct way to evaluate the column efficiency is by calculating the number of theoretical plates. At first, HPLC columns were packed only with silica and non-polar solvents such as n-hexane were delivered (normal phase). Later, the silica was modified with nonpolar compounds and typically silanes with C18 chain length (reversed phase) were applied that enabled the use of a wide range of mobile phases. The reversed phase C18 columns allowed

for more stable retention of the analytes and soon became the major separation tool since its commercial debut in 1975 (Unger *et al*., 2013). Typical C8 or C18 reversed phase columns are capable of providing more than 5000 theoretical plates (Dolan and Snyder, 2013).

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{2u}{Fk'_m}\left(\frac{k}{1+k}\right)^2 \quad (2.1)$$

Where H refers to the theoretical plate height of the column, d_p refers to the particle diameter, D_m refers to the molecular diffusion coefficient, k'_m refers to the apparent mass-transfer coefficient, k refers to the retention factor, u refers to the linear velocity of the mobile phase, and λ and γ are constants.

During a typical HPLC method development process, resolution between two adjacent peaks is a measure of adequate separation, which can be evaluated according to Equation 2.2 in isocratic HPLC (Dolan and Snyder, 2013).

$$R_S = 0.25N^{0.5}[k_1/(1+k_1)](\alpha - 1) \quad (2.2)$$

Where R_S refers to the resolution between two adjacent peaks, N refers to the number of theoretical plates, k_1 refers to the retention factor of the first peak, and α refers to the selectivity between the adjacent peaks, which can be calculated according to Equation 2.3.

$$\alpha = k_2/k_1 \quad (2.3)$$

Where k_2 refers to the retention factor of the second peak.

In ion chromatography that specifies to separate anions or cations, retention factor is a fundamental parameter to evaluate the separation, which can be calculated according to Equation 2.4 (Paull and Nesterenko, 2013).

$$k = \frac{t_R - t_0}{t_0} = D\varphi \quad (2.4)$$

Where k refers to the retention factor of the analyte ion, t_R refers to the retention time of the analyte ion, t_0 refers to the retention time of the mobile phase eluent, D refers to the distribution coefficient, and φ refers to the phase ratio.

Previously the most common HPLC detectors include ultraviolet (UV), ultraviolet visible (UV/vis) and refractive-index (RI) detectors, although the latter one has always suffered from incompatibility in gradient mode (Snyder and Dolan, 2013). Diode-array detectors (DAD) that are capable of simultaneous measurement of multiple wavelengths in the range of 185 to 365 nm

became available in the 1980s (Snyder and Dolan, 2013). One of the advantages of using a UV detector is that the detection is non-destructive, and this technique allows for detection and identification of individual compounds. The identification of glucosinolates using DAD is typically based on comparisons of the retention times and UV spectra of unknown compounds with authentic standards. Typical chromatograms are recorded at 226-229 nm or 235 nm for desulfo-glucosinolates or intact glucosinolates (Matthäusa and Zubr, 2000; Mellon *et al*., 2002; Prestera *et al*., 1996). The risk of using UV-based detection is that co-eluting compounds might interfere with analysis. The introduction of mass spectrometry (MS) as mentioned can serve as a confirmation tool to identify un-separated compounds. The coupling between HPLC and MS is becoming more popular and is conducted in routine detection of glucosinolates (Agerbirk and Olsen, 2012).

2.3.4 Quantitative analysis of glucosinolates

Methods for quantitation of glucosinolates can be divided into four categories including techniques based on measuring total glucosinolates, individual intact glucosinolates, desulfoglucosinolates, and their degradation products, as summarized by Kiddle *et al*. (2001). In an attempt to build a glucosinolates content database for edible cruciferous vegetables, McNaughton and Marks (2003) conducted a strict search into literature that provided quantitative data of glucosinolates in those plants, and three quantitative measurements were considered appropriate: analysis based on glucose released, GC (gas chromatography) and HPLC (high-performance liquid chromatography) analysis of intact glucosinolates (or desulfoglucosinolates), with the content expressed in terms of mg/100g fresh weight. Glucose-release methods determine the amount of glucose formed from the hydrolysis reaction catalyzed by myrosinase. Such methods reflect total glucosinolate content, with no further information on the individual glucosinolates being obtained (Heaney *et al*., 1988). Chromatography-based methods that allow quantitative analysis of individual glucosinolates are the most common ones. Methods based on (capillary) gas chromatography–mass spectrometry (GC-MS) were previously developed that worked to detect trimethylsilylated derivatives of glucosinolates after desulfation (Fahey, *et al*., 2001; Shaw *et al*., 1989). Drawbacks of using GC methods for individual glucosinolates analysis include tedious sample preparation, considerable matrix effect due to poor volatility of desulfoglucosinolates with specific groups, and heat sensitivity of some glucosinolates (Matthaus and Luftmann, 2000). Reversed phase HPLC (RP-HPLC) analysis is a preferred method over GC

in quantitative analysis of individual glucosinolates, since it can minimize the limitations mentioned above. The RP-HPLC method that has been in use is based on the determination of desulfoglucosinolates, which identified the compounds by their desulfated derivatives (ISO, 1992). Such methods remain the method of choice for analysis of glucosinolates from various plant extracts, while other attempts have been made to develop analytical methods for intact glucosinolates to better understand their properties, to simplify the preparation procedure and also to leave space for future bioactivity research. Glucosinolates are highly hydrophilic and non-volatile compounds due to the presence of the thioglucose moiety and the sulfate group, which contributes to the compounds' acidic property (low pKa) (Olsen and Sørensen, 1981). Their polar nature leads to poor retention in the non-polar stationary phase of RP-HPLC. The application of ion-pair chromatography is one of the commonly used methods that allow separation of intact glucosinolates (Tsao *et al*., 2002; Cai *et al*., 2004; Kaushik and Agnihotri, 1999; Jen *et al*., 2001; Prestera *et al*., 1996).

More recently, quantitative NMR (qNMR) has become a popular tool for analytical measurements of natural products, as it is fast, unbiased and informative. To accurately analyze organic compounds using qNMR, two prerequisites are required: referencing and calibration (Pauli *et al*., 2012). The first determines the chemical shifts on which the resonance of the analyte(s) can be captured, while the second conducts the quantitation. A certified internal standard that is of high purity and well characterized structure - and which is different from the analyte so as to avoid signal overlap - is needed in order to satisfy accurate quantitation (Pauli *et al*., 2012). This method is especially useful for glucosinolate measurement within the plant matrix, since for many compounds within the group, sufficient amounts of standards for chromatographic measurements are unavailable. The anionic nature of glucosinolates also implies that this kind of compounds often occurs in the form of salts, which may include a certain amount of impurities. Quantitative NMR is a suitable tool to measure the exact or close to exact amount of the target compounds in samples. Mohn *et al.* (2007) used 1,3,5-trimethoxybenzene as the internal standard to test the purity of reference glucosinolate standards by quantitative proton NMR, and revealed a significant difference in determining the true content of glucosinolates against the results from LC-MS. This study illustrated how quantitative NMR was able to examine the absolute amount of analytes in purchased and isolated samples more accurately, by compensating for the errors that occurred during chromatographic separation of

compounds with ionic nature (Mohn *et al.*, 2007).

In quantitative analysis, a calibration curve (or standard curve) is often constructed where the detector responses are plotted as a function of the known concentration of standard solutions. The amount of analyte from experimental samples can then be measured from the calibration curve. In many cases, a linear (straight) calibration curve is built by means of a procedure called "least squares", where the slope and intercept are calculated by minimizing the sum of squares of the vertical deviations (Harris, 2003).

The measurement of responses to construct a calibration curve varies between different detectors. HPLC, as described above as the most popular analytical tool to measure glucosinolates, can be connected to spectrophotometric detectors like ultraviolet (UV) absorbance detectors and fluorescence detectors, refractive index detectors, evaporative light-scattering detectors and electrochemical detectors. UV absorbance detectors based on photodiode array are most commonly used. Responses from analytes are obtained by integrating their corresponding peak area that change with concentration at specific retention times. For quantitative NMR, responses are obtained by integrating the peak area at specific chemical shifts after setting the internal standard as 1.

2.4 Glucosinolates in *Brassica carinata* and *Camelina sativa*

2.4.1 Glucosinolate profiles in *Brassica carinata* and *Camelina sativa*

The type of glucosinolates present and their concentrations vary among species within the family Brassicaceae, and even within different tissues of the same plant (Barillari *et al*., 2005; Bellostas *et al*., 2007). In addition, agronomic and environmental factors (such as moisture, temperature and soil condition), as well as different growth stages of a plant may also affect total glucosinolate content and relative proportions of individual glucosinolates (Fenwick and Heaney, 1983; Rask, *et al*., 2000; Sarwar and Kirkegaard, 1998; Schuster and Friedt, 1998). Bellostas *et al*. (2007) compared four *Brassica* species within the "Triangle of U" (*B. carinata, B. nigra, B. juncea and B. rapa*), investigating their glucosinolate profiles and concentrations in different tissues during different growing stages by measuring the desulfoglucosinolates using micellar electrokinetic capillary chromatography (MECC). Sinigrin (prop-2-enylglucosinolate) was found to be the major glucosinolate in *B. carinata, B. nigra* and *B. juncea.* In ripe seed of *B. carinata,* total glucosinolates content was 116 μmol g DM^{-1}, of which more than 100 μmol g DM^{-1} was

sinigrin (Bellostas *et al*., 2007), and because of this, an accurate measurement of sinigrin is the key to gaining information on the compounds' distribution in *B. carinata*. When sinigrin reacts with myrosinase, AITC (allyl isothiocyanate) is produced, which is a volatile oil that has a number of potential uses in agricultural and pharmaceutical industries (Sharma *et al*., 2012; Tsao *et al*., 2000). Other glucosinolates reported in *B. carinata* included gluconapin (but-3-enylglucosinolate), 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl), gluconasturtiin (phenethyl) and progoitrin (2-hydroxybut-3-enylglucosinolate), the last one of which can eventually decompose to oxazolidine-2-thiones, which are considered "antinutritional" compounds in animal feed (Bellostas *et al*., 2007; Fahey *et al*., 2001).

Glucosinolates in seed of *C. sativa* were previously identified indirectly by measuring the enzymatic hydrolysis products using GC; only two glucosinolates (9-methylsulfinylnonyl-glucosinolate and 10-methylsulfinyldecyl-glucosinolate) were identified at that time (Daxenbichler *et al*., 1991). Later, one additional glucosinolate with a similar structure was identified as 11-methylsulfinylundecyl-glucosinolate with the help of GC-MS and thermospray-MS (Lange *et al*., 1995). In the seed of *C. sativa*, the total glucosinolate content has been reported to range from 9 to 19 μmol/g (Matthäusa and Zubr, 2000), 21 to 34 μmol/g (Lange *et al*., 1995), and 13.2 to 36.2 μmol/g dry seed (Schuster and Friedt, 1998). Glucocamelinin (10-methylsulfinyldecyl-glucosinolate) is the predominant glucosinolate, accounting for 62 to 72% of the total amount, while the relative ratio of the other two glucosinolates varies among different genotypes (Matthäusa and Zubr, 2000; Schuster and Friedt, 1998). *C. sativa* glucosinolates and glucoraphanin (4-methylsulfinylbutenyl) are all methylsulfinyl glucosinolates with similar structures. Glucoraphanin - which is present in broccoli in abundance - has been extensively studied for being the precursor of the isothiocyanate sulforaphane, which is a proven potent indirect antioxidant of being the inducer of Phase II detoxification enzymes (Fahey and Talalay, 1999; Fahey *et al*., 2003; Zhang *et al*., 1992). Since these three glucosinolates found in *C. sativa* all have long side chain (Figure 2-6), the hydrolysis products should have low volatility and be comparatively stable. Few studies have been conducted on the biological activities of the hydrolysis products from *C. sativa* glucosinolates, and the lack of authentic standards of these compounds might be one of the main reasons.

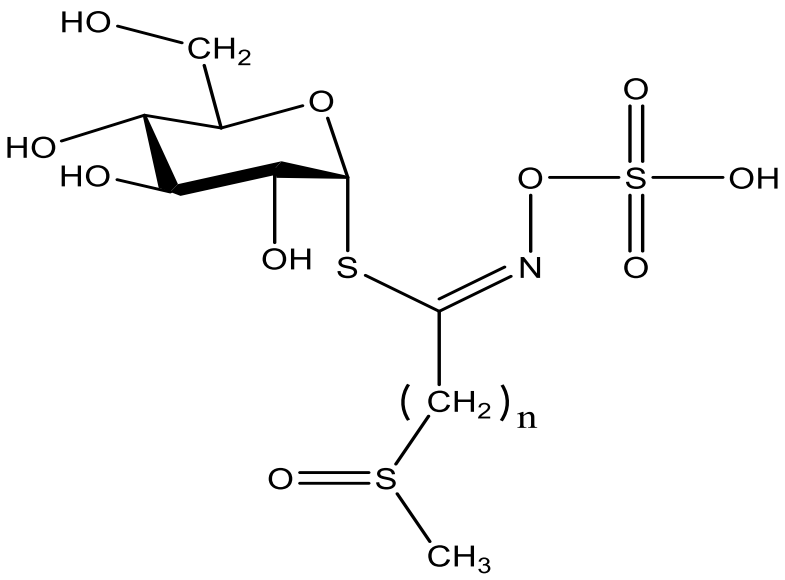


Figure 2-6 The structure of glucosinolates identified in *Camelina sativa.*

2.4.2 Measurements of glucosinolates during processing of oilseeds

Matrix effect in quantitative analysis of compounds in biological samples is important and has been discussed and reviewed a lot. Its definition in analytical chemistry, as quoted here from IUPAC is "The combined effect of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference" (McNaught and Wilkinson, 1997). In some circumstance, plant matrix can also influence the reaction of the components. In the case of glucosinolates, it was reported that plant matrix could have intense impact on the degradation of the corresponding glucosinolates (Hanschen *et al*, 2012a). The method of standard addition is often employed, to assess the "true content" of an analyte in complex matrix in an analytical procedure, where a linear response (calibration curve) is required as described in section 2.3.4. In a standard addition method, known amounts of analytes are added to the sample, which contains unknown quantities of analyte. The concentration of analyte in the samples is then calculated as $[X]_i$ based on Equation 2.5 (Harris, 2003).

$$\frac{[X]_i}{[S]_f+[X]_f} = \frac{I_X}{I_{S+X}} \tag{2.5}$$

Where $[X]_i$ refers to the concentration of analyte before spiking, $[S]_f$ + $[X]_f$ refers to the concentration of analyte after spiking (Equation 2.6), *Ix* refers to the signal response from the

solution before spiking, and I_{S+X} refers to the signal response from the solution after spiking.

$$[X]_f = [X]_i \left(\frac{V_0}{V}\right) \tag{2.6}$$

Where V_0 and V refer to the solution's volume before and after spiking, and their quotient is also called the dilution factor.

Addition of an internal standard is another approach to estimate an accurate amount of analyte in complex matrix, where known amounts of internal standard (different compounds from analyte) is added to samples with unknown amount of analyte with the assumption that the ratio factor (F) of the analyte and internal standard remains the same (Equation 2.7).

$$\frac{\boldsymbol{A_X}}{[\boldsymbol{X}]} = F\left(\frac{\boldsymbol{A_S}}{[\boldsymbol{S}]}\right) \tag{2.7}$$

Where A_x refers to the response signal from analyte, [X] refers to the concentration of analyte, A_s refers to response signal from the internal standard, and [S] refers to the concentration of the internal standard.

While enzymatic degradation of glucosinolates has been studied and discussed in great detail, the compounds are also bound to go through degradation induced by other mechanisms, including chemical degradation, thermal degradation, and the degradation that occurs within an animal's digestive system. Bones and Rossiter (2006) reviewed the enzymatic and non-enzymatic degradation of glucosinolates in detail. Deeper investigation of these non-enzymatic degradations of glucosinolates is of great importance, as it would reveal and predict the fate of glucosinolates during the processing of different plants and oilseed, and furthermore provide a picture for the possible occurrence of isothiocyanates and/or other bioactive compounds formed during the degradation of glucosinolates.

There are many factors influencing the content of glucosinolates during processing of the oilseed and plants. Because of its universal application, the influence of thermal treatment and other related factors on the degradation of glucosinolates has been an important subject of research. McNaughton and Marks (2003) concluded that cooking could result in a decrease in glucosinolate content in cruciferous vegetables ranged from 18.1 to 59.2%, according to a selection of available literature. Oerlemans *et al*. (2006) studied the thermal degradation of eight glucosinolates in red cabbage, and found that all were subject to thermal degradation upon heating the plant; from the simulation developed, canning could cause the most severe

degradation, which resulted in 73% degradation of the total glucosinolates. Mohn and co-workers (2007) found that glucosinolates were subject to loss above the temperature of 50^{o}C, and more than 60% loss occurred at 100^{o}C within a short time. On the other hand, during evaluating the thermal stability of sinigrin and sinalbin in yellow and brown mustard seed, Herzallah and Holley (2012) found that both glucosinolates were relatively stable, given a decrease of less than 10% of these glucosinolates after boiling for over 60 min. Sarvan *et al*. (2012) proposed a mathematical model that was developed through previous experimental data and that took into account several effective mechanisms, to predict the loss of glucosinolates in *Brassica* plant tissues during thermal processing, through which the level of glucosinolates were estimated to decrease after sterilization (82%), domestic boiling (69%), blanching (37%), and microwave heating (8%). Hanschen *et al*. (2012b) analyzed the content of eleven individual glucosinolates in broccoli sprouts during roasting and cooking treatment, and reported that both the chemical structures of the compounds and the pH of aqueous medium had influence on the compounds' stability during thermal treatment. It was observed that while all analyzed glucosinolates were subject to thermal degradation, the sulfur-(IV) atom in the compound and the neutral and/or acid environment comparatively stabilized the compounds (Hanschen *et al*, 2012b). A later study focusing on degradation behavior under thermal processing of five sulfur-containing aliphatic glucosinolates (methylsulfanylalkyl and methylsulfinylalkyl) revealed that nitriles, instead of isothiocyanates, were the major products upon roasting and/or cooking, and that the plant matrix environment and the addition of iron tended to increase this degradation (Hanschen *et al*, 2012a). Earlier literature discussing the more significant effect of processing on the glucosinolate content was written by Fenwick and Heaney (1983).

The means of quantitative measurement also affect the accurate determination of the true value of the compounds in plant samples. In a typical chemical analysis, a few general steps are followed, including the selection of procedures, sampling, sample preparation, qualitative and quantitative analysis of analytes from samples prepared, reporting of the results obtained, and making the conclusions (Harris, 2007). While ion suppression or ion enhancement using HPLC-MS/MS caused by co-eluting but undetected impurities in the sample have been discussed to a large extent (Matuszewski *et al*., 2003), matrix effect could in fact include many aspects.

3. MATERIALS AND METHODS

3.1 Materials and chemicals

A plot of brown-seeded *Brassica carinata* seeds were provided by Dr. Kevin Falk of Agriculture and Agri-Food Canada (Saskatoon, SK, Canada); another plot of yellow-seeded *B. carinata* seeds were obtained from Agrisoma Biosciences Inc. (Saskatoon, SK, Canada). Seeds of *Camelina sativa* were obtained from plants grown in field plots near Saskatoon, SK, Canada. Chemicals used during the conduct of this thesis research are listed in Appendix A. Deionized water (H_2O) was prepared for all mobile phases from a Milli-Q Integral system (Millipore, Bedford, MA, USA). All solvents were filtered and degassed (Teflon filter membranes, Aura Industries Inc., New York, NY, USA) prior to use in chromatography system.

3.2 Chromatographic studies of sinigrin

An Agilent series 1200 system (Agilent Technologies, Mississauga, ON, Canada) was used for all chromatographic analysis. The system was equipped with a G1311A quaternary pump, a G1322A degasser, a G1367B auto-sampler and a G1315D diode array detector. A wavelength range between 190 and 400 nm was available for UV detection. Data acquisition and analysis were performed on a ChemStation LC 3D™ software system (Agilent Technologies, Mississauga, ON, Canada). The efficiencies of two HPLC columns were compared for sinigrin analysis throughout method development and pre-validation stages. The first column was an analytical ZORBAX Eclipse XDB-C™ 18 reversed phase column (150 × 4.6 mm I.D., 5 µm particle size, Agilent Technologies, USA) packed with an Eclipse XDB C™ 18 analytical guard column (12.5 × 4.6 mm I.D., 5 µm particle size, Agilent Technologies, USA). The other was an Inertsil® ODS-4 C18 reversed phase column (150 × 4.6 mm I.D., 5 µm particle size, GL Sciences Inc., Japan) with and Inertsil® ODS-4 C18 Cartridge guard column (10 × 4.6 mm I.D., 5 µm particle size, GL Sciences Inc., Japan). A Chromolith® Performance RP-18e reversed phase column (100 × 4.6 mm I.D., 2 µm macropore size, Merck KGaA, Damstadt, Germany) packed with a Chromolith® Cartridge guard RP-18e column (5 × 4.6 mm I.D., 2 µm macropore size, Merck KGaA, Damstadt, Germany) was used to study the impact of flow rate on chromatography.

Sinigrin solution in water was used throughout the HPLC method development and pre-

validation. Sinigrin hydrate (25.0 mg) was accurately weighed using an analytical balance (Accuracy: 0.1 mg, Model: P/PI-214, Denver Instrument, Bohemia, NY) into a 25 mL volumetric flask (VWR International LLC., Arlington Heights, IL, USA). Milli-Q water was used to bring the volume to a final stock solution of 1000 µg/mL. The solution was well mixed, filtered through 0.45 µm (PTFE membrane, VWR International) with syringe (Terumo 1 ml Syringe; Terumo Medical Corp) and then transferred to a 6-dram vial (VWR International, LLC., Arlington Heights, IL, USA). Parafilm™ (Pechiney Plastic Packaging, Chicago, IL) was used to seal the vial immediately in order to limit evaporation. The stock solution was stored at 0-4^{o}C in a refrigerator (Model 153, Fisher Scientific, Dubuque, IA). On each day of analysis, standards were freshly diluted from the stock solution with Milli-Q water.

For the experiments using the Chromolith® performance column and those throughout HPLC method validation, sinigrin standards were prepared in the HPLC mobile phase from the beginning of the gradient. The preparation and storage of stock solution (1000 µg/mL, w/v) was the same as described above, with the mobile phase being used as the solvent. Standards of different concentrations were freshly diluted from the stock solution with mobile phase on each day of analysis.

3.2.1 HPLC method development for sinigrin analysis

Sinigrin stock solution (1000 µg/mL, w/v in water) was diluted to 1, 10, and 100 µg/mL in 2 mL HPLC vials (12 × 32 mm, Canadian Life Science, Peterborough, ON). Each sample was injected (10 µL) *via* the system auto-sampler and was measured three times. KNO_3 (0.1 M in Milli-Q water) was injected (10 µL) at the beginning of each solvent composition as the mobile phase void volume marker. The retention factor (k) of sinigrin was calculated according to Equation 3.1. The number of theoretical plates was calculated according to Equation 3.2.

$$k = \frac{(t_R - t_m)}{t_m} \tag{3.1}$$

Where t_R refers to the retention time of sinigrin, and t_m refers to the retention time of KNO_3 (the retention time of the void volume). Analysis was conducted at the same flow rate.

$$N = 5.545 \times \left(\frac{t_R}{W_h}\right)^2 \tag{3.2}$$

Where N refers to the number of theoretical plates, and W_h refers to the peak width at half peak

height. This value (W_h) is automatically calculated in post run analysis by the system's software.

For the selection of ion-pair reagent, water was used as the control. Ammonium acetate and ammonium formate were prepared at both 25 mM and 50 mM in Milli-Q water. Ammonium sulfate was prepared at 20 mM in Milli-Q water. Tetramethylammonium bromide (TMAB) was prepared at 5 mM and 10 mM in Milli-Q water. The aqueous ammonium buffer with acetonitrile (1%, v/v) was delivered under isocratic flow and composition at a rate of 1 mL/min on both C18 columns. Detector wavelengths for sinigrin measurements were set at 228 nm when ammonium acetate or ammonium formate was used, and 235 nm when TMAB was used (Prestera *et al*., 1996; Tsao *et al*., 2002).

Solvent optimization step included studies on the influence of buffer strength, buffer pH and percentage of organic solvent. Buffer strength was studied with TMAB prepared at concentrations of 1, 5, 10, 15, and 20 mM in Milli-Q water. The aqueous TMAB buffer with 1% of Acetonitrile was delivered to both columns under isocratic conditions. For the influence of aqueous buffer pH and organic modifier, the aqueous TMAB solution (10 mM) was adjusted to pH 3, 4, 5, and 6 using 0.1 % formic acid (v/v, in Milli-Q water) and eluted under isocratic conditions with acetonitrile (0, 0.5, 1, 5, and 10%) on both columns.

The influence of column temperature was determined by setting different temperatures (25, 35, 45, and 55^{o}C) on both columns with two solvent systems: 100% aqueous buffer (10 mM TMAB at pH 5) and 99% aqueous buffer (10 mM TMAB at pH 5) with 1% Acetonitrile.

Mobile phase flow rates were tested on both columns to optimize column efficiency (N). Flow rates at 1, 1.1, 1.2, 1.3, 1.4, and 1.5 mL/min were delivered to both columns. The isocratic solvent was 100% aqueous TMAB (10 mM, pH 5) with a column temperature set to 25^{o}C.

In addition, the isocratic eluent was delivered at flow rates of 1, 1.5, 2, 2.5, 3, and 3.5 mL/min on the Chromolith® monolithic column. For this column the temperature was set at 25^{o}C with isocratic flow of 100% of aqueous TMAB (10 mM, pH 5). Study samples included sinigrin standards prepared in Milli-Q water, and in the mobile phase (TMAB 10 mM, pH 5), both at concentrations 1, 10, and 100 µg/mL.

3.2.2 HPLC method validation for sinigrin analysis

An isocratic mobile phase of aqueous tetramethylammonium bromide (TMAB 10 mM, pH 5) delivered to the Inertsil® ODS-4 C18 reversed phase column described above was used for the

analysis of sinigrin. Column temperature was set at 25°C. The column was equilibrated with a minimum of 60 column void volumes before analysis. A flow rate of 1 mL/min was used and the column effluent was monitored at a wavelength of 235 nm with a 16 nm bandwidth against a reference at 360 nm with a 100 nm bandwidth. Injection volume for each sample was set at 20 µL during method validation. Each sample was measured three times on each day unless otherwise stated.

A pre-validation step was conducted by measuring the sinigrin standards prepared in two matrices on both C18 columns, using the optimized HPLC conditions. Stock solutions of sinigrin, prepared in the mobile phase and in Milli-Q water, were diluted concentrations that ranged between 1 and 100 µg/mL. Each sample was injected onto the HPLC column in triplicate, and the chromatographic behavior of eluting sinigrin was compared.

Validation parameters assessed included detection limit (DL) and quantitation limit (QL), linearity, precision and accuracy of the proposed HPLC method, all of whose evaluation were conducted according to the guidelines provided by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, 2005).

DL and QL were determined by injecting a series of sinigrin standards between 0.1 and 5 µg/mL. The samples were analyzed in an order of increasing concentration, and a blank run was conducted between each sequence. Each sample was measured a total of three times per day on four consecutive days. A calibration curve (n=12) was constructed in the form of Equation 3.3 using linear regression analysis. DL was calculated according to Equation 3.4 and QL was calculated according to Equation 3.5 (ICH, 2005).

$$y = mx + b \tag{3.3}$$

Where y refers to the peak area (response) generated by detector against x, which refers to the concentration of sinigrin standards injected.

$$DL = \frac{3.3\sigma}{S} \tag{3.4}$$

$$QL = \frac{10\sigma}{S} \tag{3.5}$$

Where σ refers to the standard deviation of response, and S refers to the slope (m) of the regression line from Equation 3.3.

Linearity was assessed by the construction of an external calibration curve. Stock solution (1000 µg/mL, w/v in mobile phase) of sinigrin was freshly diluted to ten concentrations between 10 and 1000 µg/mL, on each day of analyses. The sinigrin calibration curve (n=12) obtained (Equation 3.3) was the result of linear regression analysis from four days in the same week. It was used as the quantitation equation for evaluation accuracy and precision.

Accuracy and precision of the proposed HPLC method was evaluated using a set of quality control samples of sinigrin standards at the concentrations of 16, 50, 160, 250 and 500 µg/mL. Each sample was measured four times each day on four different days in the same week. On each day of analyses the samples were freshly diluted from stock. Determination of the precision involved repeatability (four consecutive injections on the same day, n=4) and intermediate precision (repeatability on four different days in the same week, n=16), both evaluated by coefficient of variation (CV%) of recovery (%). The accuracy of the proposed HPLC method was determined by calculating the recovery (%) according to Equation 3.6.

$$\%\, Recovery = \frac{calculated\ concentration}{actual\ concentration} \times 100\% \qquad (3.6)$$

Where *calculated concentration* was obtained from the external calibration curve from linearity.

3.3 Sinigrin distribution in seed of *Brassica carinata*

3.3.1 Crude oil extraction

An oilseed expeller press (Komet, type CA59C; IBG Monforts Oekotec GmbH & Co., KG, Westfalen, Germany) packed with an 8 mm choke was used to crush the *B. carinata* seed (1 kg) at a spindle speed of 62 rpm. Heat was applied on the pressing head during processing. Seeds and pressed crude oil were weighed using a balance (± 0.1 g; Explorer, Ohaus Corp., Pine Brook, NJ). Triplicate measurements were conducted for oil yield, which was calculated according to Equation 3.7. Crude pressed oil was allowed to stand for 2 days at room temperature to remove particulates by sedimentation. Upper layer oil was decanted and the sediment (a.k.a. foots) was then separated from the remaining oil by suspending into two to four volumes of hexane. This was followed by filtration through a glass-fibre filter (Whatman, Grade GF/A, Whatman Inc., Piscataway, NJ, USA) in a Buchner funnel connected to a vacuum (approximately 9,000 Pa). The separated oil foots were dried in the fume hood overnight.

$$\%\ Oil\ yield = \frac{Weight\ of\ oil\ obtained\ from\ pressing}{Weight\ of\ seeds\ pressed} \times 100\% \quad (3.7)$$

3.3.2 Oil extraction (Goldfisch)

Oil content of *B. carinata* seed materials was determined on an "as is" basis. Whole seed and seed meal from pre-pressing were ground in a coffee grinder for 30 s to pass through a 425-µm test sieve (No. 40, VWR, West Chester, PA, USA). The materials were stored in a refrigerator (4^{o}C) before analysis. The oil extraction protocol was followed based on a modified version of the method 960.39.16 from AOAC (1990a). In this method ground material (~2.8 g) was weighed, wrapped in a piece of filter paper (Whatman No. 1, 110 mm, Whatman Inc., Piscataway, NJ, USA), and folded into a cellulose extraction thimble (25 × 80 mm, Whatman Inc., Ahlstrom AT, Holly Spring, PA, USA), which was then fixed into a Goldfisch extractor (Model 35001 Labconco Co., Kansas City, MO, USA). Extraction beakers were pre-dried in a drying oven (100^{o}C) for 3 h and cooled in a glass desiccator overnight before being weighed. Hexane (40 mL) was added to each beaker, which was then attached to the lipid extractor. The extraction was carried out for 6 h at high heat. After extraction, hexane in the oil samples was recovered in a glass solvent recovery tube. Subsequently, traces of solvent in the beakers were removed by placing the beakers in oven (105°C) for 1 h. The beakers were then placed into a desiccator, and allowed to cool for at least 1 h. The mass of oil present in the beaker was used to calculate the oil content according to Equation 3.8. The weight of the beakers and beakers containing oil after drying were determined using an analytical balance (Accuracy: 0.1 mg, Model: BP221S, Sartorius, 30 South Cross Road Bradford, MA, USA). Three separate oil extractions were performed for each material.

$$\%\ Oil\ content\ = \frac{weight\ of\ beaker\ and\ oil - weight\ of\ beaker}{weight\ of\ sample} \times 100\ \% \quad (3.8)$$

3.3.3 Sinigrin extraction from solids and oil of *Brassica carinata*

Sinigrin level in *B. carinata* was measured on an "as is" basis. Ground materials were weighed (50 mg) into a centrifuge tube (2.0 mL, MCT Graduated Natural, Fisher Scientific), followed by addition of 70% (v/v) aq. methanol (1.5 mL × 8). Contents in the tubes were mixed for 2 min (× 8) at a median speed (6-8) on a test tube mixer (Analog vortex mixer, VWR International LLC., Arlington Heights, IL, USA) and centrifuged at 12,700 rpm for 10 min (× 8) (Spectrafuge™ 24D Digital Microcentrifuge, Labnet International Inc.). The supernatant was

transferred to a 5-dram vial (VWR International, USA) and the remaining pellet was extracted as mentioned above for a total of eight times. Combined supernatant (~12 mL) was concentrated under vacuum using a rotary evaporator (Rotavapor R-200, Buchi, Westbury, NY, USA) at 40°C. The concentrate was re-constituted in 3 mL of the HPLC mobile phase (TMAB 10 mM, pH 5) and 1 mL of sinigrin standards (100 - 800 µg/mL, m/v in mobile phase) to bring it to a total volume of 4 mL. Blanks were re-constituted in 4 mL of the mobile phase.

Freshly pressed *B. carinata* crude oil (50 g) was extracted with 70% (v/v) aq. methanol (1.5 L × 2) at room temperature for a total of 24 h. The combined organic layer on the top was concentrated using a rotary evaporator, and the residual oil in the concentrate was eluted with hexane until the hexane layer was clear. The dried concentrate was weighed (1 mg) and re-constituted in 0.5 mL of the HPLC mobile phase and 0.5 mL of sinigrin standards (100-800 µg/mL, m/v in mobile phase) to bring it to a total volume of 1 mL. Blanks were re-constituted in 1 mL of the mobile phase.

Extraction was performed in triplicate for whole seed and seed meal of *B. carinata* and it was performed in a non-replicated manner for the crude oil, while all samples for HPLC analysis were prepared in triplicate. Each re-constituted sample was well mixed and filtered through a 0.2 µm PTFE syringe filter (13 mm, VWR International LLC., Arlington Heights, IL, USA). A total of 20 µL from each sample was injected into the HPLC system or MS detector.

3.3.4 Quantitation of sinigrin from *Brassica carinata* seed fractions

The method of standard addition was used for determining the quantity of sinigrin in *Brassica carinata*. A calibration curve was constructed for each matrix material with a set of integrated HPLC peak areas of sinigrin plotted as the y-axis, while the concentrations of sinigrin standards in the re-constituted samples were plotted as the x-axis. The zero point, representing the amount of sinigrin in the unspiked sample (blanks), was calculated where the regression of the line intercepted to zero on the y-axis.

In order to further investigate the matrix effect during the measurement, sinigrin level in *B. carinata* whole seed was determined and compared using three methods. Three sets of samples were prepared and subject to HPLC analysis. The first set was prepared as described in section 3.3.3 and with standard solutions being added to the samples after extraction. In the second set, standard solutions were added before extraction. For sample preparation, the ground

seed was weighed (50 mg) in a centrifuge tube, followed by the addition of 0.5 mL of 70% (v/v) aq. methanol and 1 mL of sinigrin standards (100-800 µg/mL, w/v in 70% aq. methanol). Blanks that represented the unspiked samples were added with 1.5 mL of 70% (v/v) aq. methanol. After manually shaking the tubes, it was centrifuged and the supernatant transferred to a 5-dram glass vial. The pellet remained in the tubes was extracted with 70% (v/v) aq. methanol (1.5 mL × 8) by mixing for 2 min (× 8) on a test tube mixer at 2,200 rpm and centrifuged at 12,700 rpm for 10 min (× 8). The combined supernatant was concentrated as described in section 3.3.3. The concentrate was re-constituted in 4 mL of the HPLC mobile phase. The third set of samples consisted of sinigrin standards solutions (100-800 µg/mL, m/v in mobile phase) that were used to produce a curve of concentration versus detector response that would act as an external calibration curve. Matrix effect, recovery rate, and processing efficiency were calculated according to Equation 3.9, 3.10, and 3.11 (Matuszewski *et al*., 2003).

$$\%\, Matrix\ effect\ = \frac{B}{A} \times 100\% \tag{3.9}$$

Where *B* refers to the actual peak area obtained from *B. carinata* samples with sinigrin standards spiked after extraction (the first set of samples), and *A* refers to the calculated peak area of the same set of samples obtained from the external calibration curve that was constructed by the third set of samples.

$$\%\, Recovery\ rate\ = \frac{C}{B} \times 100\% \tag{3.10}$$

Where *B* refers to the same value described in Equation 3.9, and *C* refers to the actual peak areas obtained from *B. carinata* samples with sinigrin standards spiked before extraction (the second set).

$$\%\, Processing\ efficiency\ = \frac{C}{A} \times 100\% \tag{3.11}$$

Where *C* refers to the same value described in Equation 3.10, and *A* refers to the calculated peak area of the same set of samples (second set) obtained from the external calibration curve that was constructed by the third set of samples.

3.4 Detection, isolation and characterization of glucosinolates in *Camelina sativa*

3.4.1 Sample preparation

Cold-pressed *C. sativa* crude oil was allowed to sit for 2 days, in order for sedimentation of oil solids. Processing of oil solids was the same as described in section 3.3.1. For identification of glucosinolates and isolation using semi-preparative LC, dry oil foot (oil solids, 0.5 g) was extracted with 80% (v/v) aq. ethanol (5 mL) in a 15 mL centrifuge tube (VWR International LLC., Arlington Heights, IL, USA). The tube was put onto a test tube mixer set at speed 8 (Analog vortex mixer, VWR International LLC., Arlington Heights, IL, USA) and mixed for 2 min, then was centrifuged at room temperature at 12,500 rpm for 20 min (Model J-E, JA-10 Rotor, Beckman Coulter, Inc., Palo Alto, CA, USA). The resultant supernatant was collected and concentrated and the concentrate was re-constituted in the starting solution for gradient HPLC chromatography (1 mL) containing 97% H_2O with 0.1% formic acid and 3% acetonitrile with 0.1% formic acid.

For preparative HPLC, the dry oil foot (0.5 g) was extracted by stirring for 72 h with 80% (v/v) aq. ethanol (30 mL) in a beaker at room temperature. After stirring the extract was centrifuged at 12,500 rpm for 20 min. The resultant supernatant was collected, concentrated and further re-constituted in the starting solution for gradient HPLC chromatography (4 mL) containing 97% H_2O with 0.1% formic acid and 3% acetonitrile with 0.1% formic acid.

All samples were well mixed and filtered through a 0.2 μm PTFE syringe filter (13 mm, VWR International LLC., Arlington Heights, IL, USA) before injecting into chromatography system.

3.4.2 Liquid chromatography conditions

Semi-preparative reversed phase HPLC was performed on an Agilent series 1200 system as described in section 3.2. A Chromolith® SemiPrep RP-18e column (100 × 10 mm I.D., 2 μm macropore size, Merck KGaA, Damstadt, Germany) set at 25°C was used for isolation of glucosinolates from *C. sativa* crude extract, with an injection volume of 96 μL for each run. The binary mobile phase consisted of (A) H_2O (with 0.1% formic acid) and (B) acetonitrile (with 0.1% formic acid) at a flow rate of 3.7 mL/min. The gradient started at 97%/3% A/B, and increased linearly to 50%/50% A/B in 8 min. The solvent composition then returned to starting condition (97%/3% A/B) in 0.5 min, and was then held at this composition for another 1.5 min.

The gradient and equilibration required 10 min for each injection. UV absorbance was monitored at a wavelength of 242 nm with a 20 nm bandwidth against a reference at 300 nm with a 10 nm bandwidth. Data analysis was performed using ChemStation for LC 3D™ system software as described in section 3.2.

Preparative reversed phase chromatography was performed on a BioCAD SPRINT Perfusion Chromatography Workstation (Perspective Biosystem Inc., MA, USA). An Inertsil® Prep-ODS column (250 × 30 mm I.D., 10 μm particle size, GL Science Inc.) was used with an injection volume of 1.9 mL for each run. The binary mobile phase consisted of (A) H_2O (with 0.1% formic acid) and (B) acetonitrile (with 0.1% formic acid) delivered at a flow rate of 7.0 mL/min. The gradient started at 97%/3% A/B then after 1 min increased linearly increased to 50%/50% A/B in 30 min. After completion of the gradient the solvent composition was returned to 97%/3% in 2 min and was then held for another 10 min to equilibrate for the next injection. The method required 43 min for each injection. HPLC effluent absorbance was monitored at a wavelength of 228 nm and 242 nm using a UV/VIS detector. Fractions were analyzed by LC-MS and suitable fractions were concentrated as reference compounds.

3.4.3 HPLC-ESI-MS analysis

HPLC-ESI-MS analysis was performed on the Agilent HPLC 1200 series system described in section 3.2, which was connected to a MicrOTOF-Q II Mass Spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an electrospray ionization source. A Chromolith® FastGradient RP-18e column (50 × 2.0 mm I.D, 2 μm macropore size, Merck KGaA, Darmstadt, Germany) was used and set at 25°C. The binary mobile phase consisted of (A) H_2O (with 0.1% formic acid) and (B) acetonitrile (with 0.1% formic acid) delivered at a flow rate of 0.4 mL/min. The gradient started at 97%/3% A/B and linearly increased to 50%/50% A/B in 8 min, then to 90%/10% A/B in 4 min followed by a hold period of 2 min. The starting composition of solvents 97%/3% A/B were restored with a 2 minute gradient, and then held for 4 min before additional injections. MS detection was performed using the negative ion mode. Other MS acquisition parameters were as follows: capillary voltage = 3500 V; nebulizer gas (nitrogen) = 4.0 bar; dry gas flow rate = 8.0 L/min; source heater dry gas temperature = 200°C; transfer time = 70.0 μs. Mass range from 50 to 1000 m/z was scanned. For MS/MS multiple reaction monitoring (MRM) analysis, three parent ions were monitored at m/z 506, 520, and 534, with the collision energy set

at 21, 22, and 22 eV respectively, and the isolation width all set at 30.

The ratio of individual glucosinolates was determined using a Chromolith® Performance RP-18e reversed phase column (100 × 4.6 mm I.D., 2 μm macropore size, Merck KGaA, Damstadt, Germany) packed with a Chromolith® Cartridge guard RP-18e column (5 × 4.6 mm I.D., 2 μm macropore size, Merck KGaA, Damstadt, Germany). The flow rate was set to 0.8 mL/min with the same chromatography gradient program as that delivered to the FastGradient column described above. In order to maintain an efficient and stable ionization, nebulizer pressure was set to 6.0 bar, dry nitrogen gas flow set to 10.0 L/min and drying gas temperature set to 220^{o}C.

3.4.4 Nuclear magnetic resonance analysis

NMR analysis was performed on a Bruker Avance spectrometer at 500.3 and 125.8 MHz for ^{1}H and ^{13}C, respectively (Inverse triple resonance probe, TXI, 5 mm; XWIN-NMR 3.5 software).

Total glucosinolates in *C. sativa* extract was determined using *N,N*-dimethylformamide (DMF) as the internal standard. DMF (2.5 mg; Model: P/PI-214, Denver Instrument, Bohemia, NY) was dissolved in 50 mL D_2O to give a concentration of 0.05 mg/mL. Each sample was subject to ^{1}H NMR analysis, with 128 scans generated. Chemical shifts (δ) values were reported in parts per million (ppm) referenced to D_2O at 4.7 ppm. The proton resonance at 2.56 ppm relative to the internal standard at 7.7 ppm was integrated as a measure of total glucosinolates.

3.4.5 Quantitation of individual glucosinolates from camelina fractions

All *C. sativa* seed fractions were subject to both NMR and LC-MS analysis. Quantitation of total glucosinolates was achieved by quantitative NMR analysis as described in section 3.4.4. The amount of individual glucosinolate in each sample was determined by measuring the integrated area from each extracted ion from the total ion in mass spectra.

3.5 Distribution of glucosinolates during processing of *Camelina sativa* seed

3.5.1 Dehulling of camelina seeds

C. sativa seeds were ground in a tabletop disc mill (C/11/1, Glen Mills Inc. Clifton, NJ, USA) that was adjusted so that most seed was cracked but not powdered. Hull was separated from the ground seed by aspiration in a vertical cylindrical seed cleaner that was attached to a

small size column blower, on top of which were the shelters that were designed to collect the blown hull and other fine materials connected to a small cylindrical column (made in Grains Innovation Laboratory of Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada).

3.5.2 Crude oil extraction

C. sativa seed crude oil pre-pressing, the handling of oil solids and the calculation of oil yield (%) were the same as described in section 3.3.1.

3.5.3 Oil extraction (Goldfisch)

Oil content of *C. sativa* seed materials were measured on an "as is" basis as described in section 3.3.2.

3.5.4 Determination of moisture content

Moisture content of *C. sativa* seed materials was determined based on a modified version of the AOAC method 950.46 (AOAC 1990b). Aluminum pans were pre-dried in an oven (102^{o}C) for 3 h, and then transferred and cooled in a glass desiccator overnight, before being weighed. Ground *C. sativa* materials were weighed (~3.2 g), spread evenly in the pan, and dried in an oven (102^{o}C) overnight (~19 h), and then transferred and cooled in a glass desiccator overnight, before being weighed. Moisture content (%) was calculated according to Equation 3.12.

$$\%\, Moisture\ content = \frac{Weight\ of\ sample - Weight\ of\ dried\ sample}{Weight\ of\ sample} \times 100\% \quad (3.12)$$

3.5.5 Crude protein content determination

The crude protein content of *C. sativa* seed materials was determined on an "as is" basis. The determination of total nitrogen content was based on a modified version of the AOAC method 990.03 (AOAC, 1997). The measurement was conducted on a LECO FP-528 (LECO Corporation, 3000 Lakeview Ave. St. Joseph, MI, USA). The crude protein content was calculated according to Equation 3.13.

$$\%\, Crude\ protein = \%\, N \times F \quad (3.13)$$

Where N refers to the nitrogen content from the combustion method and F refers a universal conversion factor of 6.25.

3.5.6 Camelina glucosinolate extraction and quantitation

Whole seed, seed meal, defatted seed, defatted meal and crude oil solid of *Camelina sativa* were all subject to measurements of glucosinolates on an "as is" basis. Materials were weighed (50 mg) in a centrifuge tube (2.0 mL MCT Graduated Natural, Fisher Scientific) followed by the addition of 80% (v/v) aq. ethanol (1.5 mL). The tube was put onto a test tube mixer set at speed 8 (Analog vortex mixer, 58816-121, VWR International, LLC., Arlington Heights, IL, USA) and mixed for 2 min, and centrifuged at 12,700 rpm for 10 min. The supernatant was transferred to a 5-dram glass and the remaining pellet was extracted for two more times as described above. The combined supernatant was concentrated under vacuum using a rotary evaporator (Rotavapor R-200, Buchi, Westbury, NY, USA) connected with at 40°C. The concentrate was further dried under vacuum pressure (Welch® 8904, Gardner Denver Thomas, Inc. Welch Vacuum Technology, Niles, IL, USA) connected to immersion cooler (Model 1109, VWR International LLC., Arlington Heights, IL, USA). The concentrate was re-constituted in DMF (0.05 mg/mL in D_2O, 1 mL) and analyzed by ^1H NMR (128 scans). Freshly pressed *C. sativa* crude oil (500 g) was extracted with 80% (v/v) aq. ethanol (1.4 L, twice) overnight. The combined organic layer (on the top layer) was concentrated using a rotary evaporator, and the residual oil was then washed again with hexane, until the hexane layer was clear. The concentrate was re-constituted in DMF (0.05 mg/mL in D_2O, 8 mL) and analyzed by ^1H NMR (128 scans). Triplicate extractions were performed for each fraction.

To evaluate the accuracy of the NMR method in quantitation, glucoraphanin standard solution (1 mg/mL in DMF (0.05 mg/mL in D_2O) was spiked into the re-constituted sample of *C. sativa* whole seed extract, for a total of four times (0.5 mL per time). The spiked samples were analyzed on ^1H NMR.

3.6 Statistical analysis

Data analysis was performed using Excel (Excel® for Mac 2011 version 14.0.0, Microsoft Corporation) and SPSS software (IBM SPSS Statistics 21). General calculations of the average, standard deviation, and coefficient of variations (CV%) were performed on Excel. Calibration curves were built using the Linest function, which determined the quantitation equation.

During HPLC method development for sinigrin, between subjects factorial analyses of variance (ANOVA) were conducted on SPSS to analyze the effect of different HPLC conditions

(factors) on the retention of sinigrin (*k*), with the significant difference level of 0.05. In order to further determine the regression model in which the retention of sinigrin (*k*) could be explained by several HPLC variables that had been proved significant from the ANOVA test, Pearson correlation and the modeling technique of standard linear regression with the enter method were applied, and the entry limit of the probability of F value was defined at 0.05. Model summary of the factors that resulted in the highest correlation was presented.

4. RESULTS AND DISCUSSION

4.1 HPLC method development strategy for sinigrin analysis

There are reported HPLC methods in peer-reviewed journals that described the HPLC analysis of sinigrin. Close examination and testing of these methods quickly eliminated each of them for reliable separation and quantitation of the compound. Primarily the methods showed poor reproducibility as they were executed on reversed phase media in current work. Although it was not reported, the columns and gradients used were unlikely to be reliable due to the gradients reported (Bidlingmeyer and Broske, 2004). Therefore, it was necessary to develop an improved analytical method for sinigrin.

Several parameters that would indicate the chromatographic behavior of analytes were assessed, such as retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N). As sinigrin was the major analyte in current work, the goal of method development was to develop a method that would afford adequate separation between this polar compound and other less-retained materials that might interfere with quantitative analysis. In an optimal situation, a k value in the range of 2 to 10 is preferred where adequate separation is achieved with a convenient analysis time, while in real-time operation a k value in the range of 1 to 20 is considered acceptable (Dolan and Snyder, 2013). Two C18 columns were selected for method development, both of which are described by the manufacturers to be useful for separating polar compounds in reversed phase chromatography.

During method development, multivariate strategy was applied to assess the influence of several HPLC conditions had on the retention of sinigrin. In biotechnology, the response surface methodology (RSM) are often used as a tool to assess the influence of multiple variables on the key response in a biological process using a range of experimental techniques and statistical analysis, from which a mathematic model (Equation 4.1) will be generated that helps to find and further predict the optimal condition to operate the process (Baş and Boyacı, 2007).

$$\eta = f(x_1, x_2, \ldots, x_n) + \varepsilon \tag{4.1}$$

Where η refers to the response, f refers to the functional relation between η and the variables $x_1 \ldots x_n$, and ε refers to the statistical error (Baş and Boyacı, 2007).

Normally, two stages of experiments are conducted before the response surface can be

demonstrated, and the first stage is a screening experiment from which several variables with significant effect would be determined to possess the second stage of experiment (Baş and Boyacı, 2007; Kalil *et al*., 2000). A previous publication (Dolan and Snyder, 2013) summarized the variables that might possess significant effect on the retention of sinigrin in the current investigation were simulated that allowed the optimization in current work be conducted from the second stage, and these HPLC variables were buffer strength, concentration of organic modifier, column temperature, column type, and mobile phase pH. The goal of the current experiment also determined the inclusion of one more HPLC variable, which was the selection of an ion-pair reagent. In addition, the three levels of concentrations of sinigrin standards in fact served as another variable. Factorial experiments are often designed as the second stage during the processing of RSM, in which variables are cross-examined at different levels. It was noted by Dolan and Snyder (2013) though, that the HPLC variables that define the chromatographic condition as listed above, had different level of influence (ranking) on the selectivity. It was also concluded from preliminary experiments in current work that a small change in some of the variable would cause a drastic change in the retention of sinigrin; and the range of some variables would only be determined after a prior result was obtained from the test of other variables. Based on this, a complete or full factorial experiment that includes all the variables would not only be time-consuming, but also lead to inaccurate conclusion. Instead, HPLC method development conducted in several factorial experiments within which more than two levels were contained in many factors would be more meaningful.

4.1.1 Determination of mobile phase volume

The retention factor *k* of sinigrin served as an indicator of the system suitability and the efficiency of a column in separating the compound during method development, since without appropriate retention, meaningful chromatography is impossible. The accurate determination of the column mobile phase void volume, i.e., the volume of an unretained compound, was therefore important as shown in Equation 3.1. Several methods of measuring the column void volume (or void time) in single or multi-component mobile phase have been described and proposed, but there has not been a universal method for this parameter, mostly because it is difficult to define a clear boundary between the stationary and mobile phases (Engelhardt *et al*., 1984; Melander *et al*., 1983; Rimmer *et al*., 2002). Pyconometry of dry media can measure a maximum column void volume, however, the column void volume is decreased by the solvation

of the stationary phase (Rinmmer *et al*., 2002). An absolute unretained compound ($k = 0$) as the void volume marker is yet to be discovered. Meanwhile, concentrated inorganic salts, such as those with NO_3^-, NO_2^-, Br^- and $Cr_2O_7^{2-}$, were concluded to be suitable void volume markers in many published work, and amongst them, nitrate ion (NO_3^-) was studied and recommended the most (Berendsen *et al*., 1980; Melander *et al*., 1983; Oumada *et al.* 2000; Rimmer *et al*., 2002). As a preliminary experiment, 0.1 M KNO_3 was injected into the RP-HPLC system with the mobile phase ranging from 0 to 100% acetonitrile with Milli-Q water. Retention times of the compound at each treatment were measured in triplicate and the means are presented in Table B-1 (Appendix B). The retention times in both columns reached the lowest when the percentage of acetonitrile was around 50-60%, where the stationary phase might be going through a process from wetting to unwetting and the compound may have been excluded by negative adsorption, as explained by Engelhardt *et al.* (1984) and referred to as Donnan exclusion by Rimmer *et al.* (2002), but the authors also addressed that this effect could be prevented when buffered mobile phase was used. This retention phenomenon was more pronounced in the ODS-4 column: the change in KNO_3 retention times presented a "U" shape, where the values were higher at 0% and 100% acetonitrile and lower at 50-60%. CVs (%) obtained were less than 0.2% for all measurements, indicating the compound's (0.1 M KNO_3) stable performance under different conditions.

The retention time of a marker was dependent on mobile phase buffer as expected for anionic analytes (Oumada *et al.*, 2000). According to this and the experimental results obtained, to measure the actual mobile phase volume under a specific condition other than the universal void volume should be more meaningful. Considering the anionic nature of sinigrin and the strategy to add specific ion-pair reagents during the HPLC analysis, 0.1 M KNO_3 was employed as the mobile phase volume marker and injected into the system at the beginning of every analysis under several HPLC conditions and their retention times were determined as the column void volume for that specific condition.

4.1.2 Selection of an ion-pair reagent

The challenge of separating glucosinolates with RP-HPLC lies in the compounds' hydrophilic nature due to the attachment of a sulfate group. Sinigrin, as a typical glucosinolate with high polarity, is not easily retained on the hydrophobic octadecyl-silica (C18) bonded stationary phase. There are two major types of RP-HPLC methods in determining individual

glucosinolates, and the common one is to hydrolyze the sulfate group by conducting a desulfation step (ISO, 1992). As reviewed in section 2, the inclusion of a desulfation step brought up various uncertainties into the final quantitation, which would lead to underestimation or overestimation of the amounts of compounds within plant matrix. Another option is to bind the intact glucosinolates using an ion-pair reagent (Sørensen, 1990). The latter method is also referred to as ion-pair chromatography (IPC), and the theory is to combine the analyte(s) with a counter ion of opposite charge to form an ion pair that is retained on the relatively nonpolar bonded phase (García-Álvarez-Coque and Torres-Lapasió, 2013). The current project adapted the second method. Szmigielska and Schoenau (2000) proposed this ion-pair step be conducted during the extraction as well as in analytical measurement stage, with the help of anion-exchange membranes. More often, ammonium salts are used as ion-pair reagents in HPLC mobile phases for this purpose. The ammonium ion can be adsorbed onto the stationary phase and then provides an ion-exchange site for glucosinolates. While there are considerable publications regarding the HPLC methods for glucosinolates, there is hardly a systematic review on comparison of the reagents' efficiency in their ion-pair strength.

In the current study, some of the previously reported approaches were further examined and compared. Ammonium acetate (Tsao *et al*., 2002), ammonium sulfate (Kaushik and Agnihotri, 1999), ammonium formate (Troyer *et al*., 2001) and tetramethylammonium bromide (TMAB) (Prestera *et al*., 1996) were selected from the literature as the ion-pair reagents for separating sinigrin in HPLC. Ammonium sulfate (20 mM) was previously reported to act as the ion-pair reagent for analysis of intact glucosinolates (Kaushik and Agnihotri, 1999). In a preliminary experiment, however, the solubility of this salt in water was not high enough to avoid clogging in the LC system and high column backpressure. This solvent was considered incompatible with the system. For ionic compounds, manufacturers recommend using a buffer in the concentration range of 10-50 mM with a C18 column. For consistency, ammonium salts were prepared at concentrations of 25 mM and 50 mM and TMAB was prepared at 5 mM and 10 mM. Mobile phase (99%/1%, A/B) was delivered under isocratic conditions. Acetonitrile was chosen as the organic solvent over others for the capability to improve peak shape and resolution (Bjerg and Sorensen, 1987).

Sinigrin retention is very poor when water is used without any ion-pair reagent (Table B-2, Appendix B). Under this condition on the ODS-4 C18 column, the compound eluted even

earlier than KNO_3. The use of ammonium acetate (25 mM) was previously reported in a published method, in which the binary mobile phase of the buffer and acetonitrile could separate sinigrin and its major hydrolysis product AITC simultaneously (Tsao *et al*., 2002). The retention factor of sinigrin was less than 1 when the gradient of Tsao *et al*. (2002) was applied in the current study. By increasing the buffer's concentration to 50 mM, a *k* value of 1 was achieved only on the Eclipse C18 column, but this LC condition was not able to measure sinigrin at a low concentration (1 μg/mL). Ammonium formate (30 mM) combined with acetonitrile as the mobile phase has been used to separate eight glucosinolates (including sinigrin) using a relatively polar stationary phase (a polyhydroxyethyl aspartamide column), and therefore the system should be considered as hydrophilic interaction liquid chromatography (Troyer *et al*., 2001). In the current study the addition of ammonium formate salt at two concentrations was able to achieve a similar retention on the Eclipse C18 column, but the *k* value was less than 1 on the ODS-4 C18 column with this gradient. Only buffer containing TMAB afforded a retention factor above 1 for sinigrin on both columns. TMAB was one of the reagents suggested for measurement of glucosinolates in a previous study, in which the buffer (5 mM) was used to separate glucoiberin and glucoraphanin; other salts were also utilized, including tetraoctylammonium (TOA) and tetradecylammonium (TDA) (Prestera *et al*., 1996). Analysis of variance revealed that not only the type of ion-pair reagent, but also different C18 columns as well as the concentration of sinigrin standards had a significant main effect ($p < 0.05$) and interaction effect ($p < 0.05$) on the retention (*k*) of sinigrin (Table B-7, Appendix B). Since TMAB was shown more lipophilic and favorable for the polar sinigrin, it was chosen as the ion-pair reagent for further optimization.

4.1.3 Influence of buffer strength

One of the advantages of ion-pair chromatography is that the operator can control the ion-pair capacity on the column by adjusting the composition of the mobile phase. Theoretically, the retention of sinigrin increases at a higher TMAB concentration in the mobile phase, as this provides more "sites" to exchange for the sinigrin ion, until the surface of the stationary phase is saturated with TMAB ions. On the other hand, the retention is expected to decrease with an increased addition of organic solvent, which accelerates the removal of the adsorbed ion pair. Under a well-developed HPLC condition, the ratio of TMAB and acetonitrile in the mobile phase for the analyte sinigrin should be kept constant to maintain a reproducible retention for further

quantitation. The influence of buffer strength on the chromatographic behavior of sinigrin is presented in Table B-3 (Appendix B). The value of HPLC parameters being assessed (t_m, t_R, k, etc.) all tended to increase with higher buffer strength. The most significant change in retention occurred when TMAB was first added into the mobile phase compared to when only water was used, which proved the ability of the ion-pair reagent on retaining the polar compound with a reproducible manner. At 10 mM of TMAB, the retention factor of sinigrin achieved 1 on both columns. Overall, the two columns showed a similar reaction to the change in TMAB concentration.

Analysis of variance was conducted to determine the influence of buffer strength (measured as the concentration of TMAB water solution), C18 columns used, as well as the concentrations of sinigrin standards on the retention of sinigrin (k). Table B-7 (Appendix B) shows that these factors all had significant main effect ($p < 0.05$) and interaction effect ($p < 0.05$) on the retention of sinigrin. For the ODS-4 column, however, higher buffer concentrations than 10 mM didn't contribute to increased retention of the compound; instead, the k value dropped. One possible explanation might be that for the ODS-4 column, when the TMAB concentration was above 10 mM the bonded phase was saturated for the pairing with sinigrin, and this interaction was meanwhile interfered with another interaction that took place. Jen *et al*. (2001) also noticed a similar phenomenon while using a reversed phase Nucleosil C18 column in separating sinigrin, although the threshold concentration they observed was around 40 mM.

Considering all chromatographic parameters assessed in the conditions tested, TMAB at a concentration of 10 mM was determined to be the appropriate aqueous buffer for further study of solvent optimization.

4.1.4 Influence of buffer pH and organic modifier

In addition to the buffer strength of the mobile phase, the retention of ionizable analytes in reversed phase chromatography is also dependent on the pH of the aqueous mobile phase and the percentage of organic solvent in the mobile phase. During method development, the impact of these two factors was investigated on sinigrin chromatography. As for silica-based columns, a pH that is too low (< 2) may lead to the hydrolysis of the bonded phase, while a pH that is too high (> 8) is likely to result in the dissolution of silica (Dolan and Snyder, 2013). In current work, pH at the range of 3 to 6 was tested. In conventional HPLC method development, the influence of organic solvent would be investigated in the range of every 10%, while the range of

acetonitrile to be tested was set at 0, 0.5, 1, 5, and 10% based on observations of the early stages of method development.

Low concentration (1 μg/mL) of sinigrin was not measurable under the following conditions: TMAB (10 mM) solution adjusted to pH 3 and eluted with 0.5, 1 and 10% of acetonitrile on the Eclipse column; TMAB (10 mM) solution adjusted to pH 4 and eluted with 10% of acetonitrile on the Eclipse column; and TMAB (10 mM) solution adjusted to pH 3 and eluted with 10% of acetonitrile on the ODS-4 column. Under these conditions the signal from the compound (1 μg/mL) was too weak for quantitative analysis or the peak was not sufficiently reproducible for integration. One possible explanation might be that at a lower pH, the ionization of sinigrin was suppressed, and the compound eluted as non-retained material without pairing with TMAB. Retention times for both the void volume marker and analyte were generally longer on the ODS-4 column than on the Eclipse column. While the retention time of nitrate decreased slightly with the increase of acetonitrile in the mobile phase, a larger decrease of the retention time for sinigrin was observed with additional acetonitrile. The retention factor of sinigrin was above 1 when the acetonitrile concentration was 0-1%. When the acetonitrile increased to 5-10%, the retention factor dropped below 0.5. This phenomenon occurred at all pH values on both columns. Repeatability of each of the condition was good, considering that all of the CV (%) for retention time fell below the acceptable 2% (Table B-4, Appendix B).

Sinigrin retention was more sensitive to buffer pH changes on the Eclipse C18 column than on the ODS-4 C18 column, while the latter showed more sensitivity to the change in acetonitrile concentration. The sinigrin retention on both columns was higher when the buffer pH was adjusted to 5 than other pH values. Also, column performance was the best at this specific pH, as indicated by the plate count (*N*). When a higher percentage of acetonitrile (10%) was applied though, almost no retention for the analyte was achieved. The addition of acetonitrile as the organic modifier was therefore contradictory: while 100% of the aqueous buffer would achieve a more favorable retention for the compound sinigrin, the inclusion of acetonitrile at a certain level improved column performance (Fig 4-1). The plate counts (*N*) were much higher for the ODS-4 column. It might bring up more investigation on the contribution of acetonitrile, with whose inclusion the overall mobile phase pH is prone to increase and the pKa of the analyte tends to decrease, as demonstrated by McCalley (2000). To rule out the influence of acetonitrile, one could observe the change in the chromatographic behavior of sinigrin as the aqueous mobile

phase was adjusted to different pH when acetonitrile was not included. For the Eclipse column, t_m remained stable with the change in aqueous buffer pH, while the retention of sinigrin tended to decrease when pH of the buffer was raised.

Analysis of variance showed that the pH of the aqueous buffer, the percentage of acetonitrile added as well as the C18 columns used and concentrations of sinigrin standards all had a significant main effect ($p < 0.05$) on the retention of sinigrin (Table B-7, Appendix B). While most of them had significant interaction effect ($p < 0.05$) on the compound's retention, there were two exceptions, which were 1) the interaction between sinigrin concentration, buffer pH, and percentage of acetonitrile (p = 0.159); and 2) the overall interaction between the four factors tested (p = 0.106).

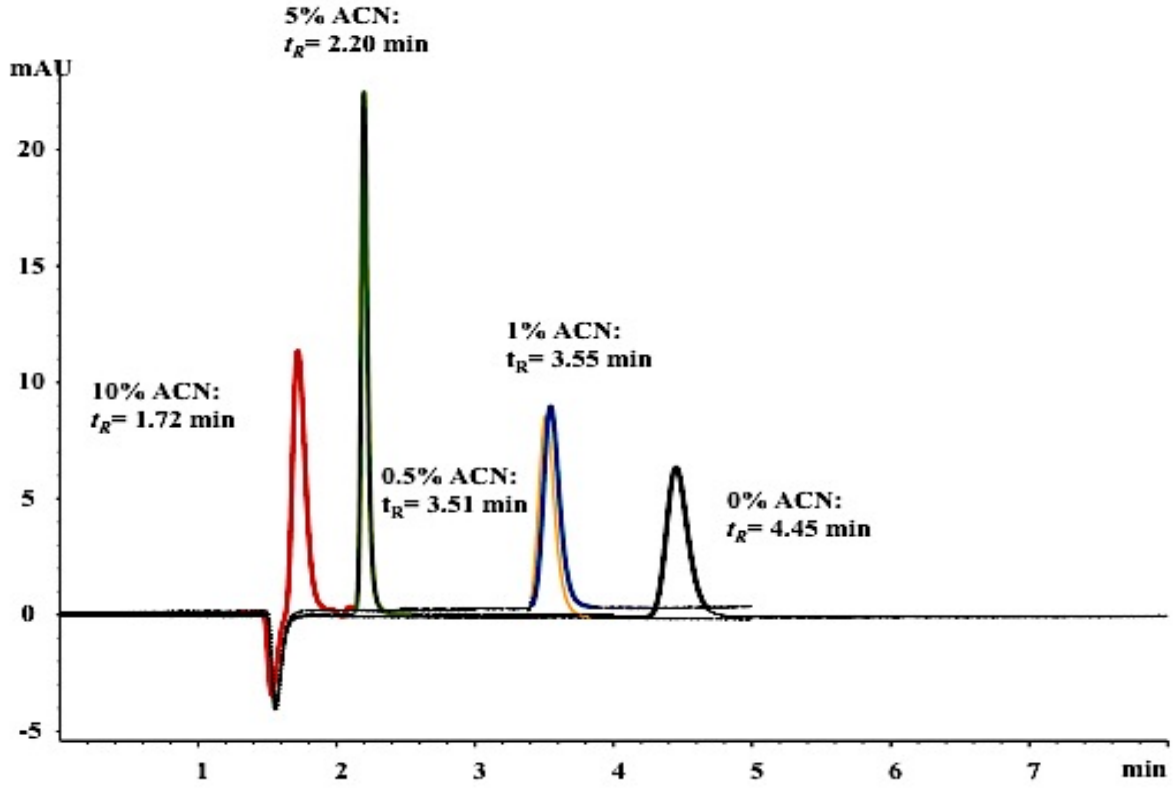


Figure 4-1 HPLC chromatogram of sinigrin (10 µg/mL) eluted with TMAB (10 mM, pH 5) at different acetonitrile concentration on Eclipse C18 column.

4.1.5 Temperature as a variable

The goal of this section was to determine an appropriate temperature that could result in

shorter analysis time while stable retention for the compound as well as high column efficiency (theoretical plates) would be maintained. As with ion-pair chromatography, it is necessary to conduct the HPLC analysis under a well-controlled column temperature that contributes to reproducible results. At the earlier method development stages described above, column temperature was not defined (with a room temperature varied around at 23^{o}C). In current work, column temperature as a variable was tested between 25 and 55^{o}C for its influence on chromatographic behavior of sinigrin. Further increase in column temperature (60^{o}C) would degrade the silica, as suggested by the manufacturer.

Table B-5 (Appendix B) is the summary of the chromatographic behavior of sinigrin with the change in column temperature. The mobile phase void times (t_m), sinigrin retention times (t_R), retention factors (k), peak width at half peak height (W_h) and number of theoretical plates (N) all tended to decrease with an elevated column temperature. Also, including 1% acetonitrile in the mobile phase lowered retention, while the difference in retention caused by the inclusion of acetonitrile tended to narrow as the temperature increased. The decrease in retention of sinigrin and the plate count with temperature was greater with the Eclipse column: the N value decreased with temperature, achieved the highest at 25^{o}C, reached the lowest at 55^{o}C in aqueous media without acetonitrile; when acetonitrile was included, a column temperature of 35^{o}C produced the highest N value. For the ODS-4 column, column efficiency was consistent with the change of column temperature and the addition of acetonitrile. Overall, the ODS-4 C18 column achieved higher column efficiency than the Eclipse C18 column. The best retention of the compound, as well as highest column efficiency, was achieved on the ODS-4 column at 25^{o}C with 0% acetonitrile.

Analysis of variance of the column temperature, the inclusion of acetonitrile in the mobile phase, C18 columns used as well as the concentrations of sinigrin standards indicated that these factors all had significant main effects ($p < 0.05$) on the retention of sinigrin. While many of the interaction effects were not significant ($p > 0.05$), the interaction between C18 columns and column temperature, C18 columns and the inclusion of acetonitrile, column temperature and the inclusion of acetonitrile, and the interaction between C18 columns used, sinigrin standard concentrations and the inclusion of acetonitrile were statistically significant (Table B-7, Appendix B).

Column temperature is a well-recognized factor in HPLC analysis, which may affect the

thermodynamic and kinetic equilibrium inside the column. An elevated column temperature can help to increase the solubility and diffusivity. In this study, the adsorption process of the ion-pair was compromised with higher temperature, which explained why the retention of sinigrin tended to decrease with raised temperature. It also provided the possible answer to the inconsistent *N* value calculated on the Eclipse column.

4.1.6 Influence of column flow rates

While many have been done previously on glucosinolates analysis using HPLC, little has been carried out regarding the effect of column flow rate on the chromatographic behavior of the compounds. The flow rate of both columns during the method development above was set at 1 mL/min as default. It was recommended by Unger *et al*. (2013) that for columns with an internal diameter 4.6 mm, the optimum flow rate is 1.3 mL/min. The optimum mobile phase flow rate might vary with different LC conditions on different columns with the same internal diameter; therefore, a range of flow rates between 1 and 1.5 mL/min were tested on both columns using the LC conditions optimized as mentioned above.

The number of theoretical plates (*N*) is a measure of the column efficacy and performance under specific conditions. A larger *N* value indicates a stronger resolving power and therefore, the efficiency of the column at separating the analyte. Both columns tested were typical reversed phase C18 columns that had broad uses in research labs. They both showed a general decrease trend in the *N* value with increased flow rate. For the Eclipse column, a flow rate of 1.1 mL/min helped to give the highest retention of sinigrin with a shorter analysis time (4.15 min). For the ODS-4 column, flow rates of 1.0 mL/min and 1.2 mL/min both achieved the highest retention of the compound, while the analysis time was shorter (4.75 min) with a flow rate of 1.2 mL/min. The highest column efficiency for analysis of sinigrin was observed at a flow rate that remained at 1 mL/min (Table B-6, Appendix B).

Analysis of variance revealed that the different C18 columns, the concentrations of sinigrin standards as well as the mobile phase flow rate delivered all had a significant main effect ($p < 0.05$) and interaction effect ($p < 0.05$) on the retention (k) of sinigrin (Table B-7, Appendix B). An optimized reversed phase condition for sinigrin was determined for both C18 columns, which involved 100% aqueous TMAB (10 mM, pH 5) with a column temperature set to 25^{o}C. The flow rate was set as the default at 1 mL/min.

4.1.7 Prediction of sinigrin retention

A systematic approach to RP-HPLC analysis of sinigrin was undertaken. Chromatographic variables including suitable ion-pair reagents, buffer strength, buffer pH and organic modifier as well as some of the less studied parameters such as column temperature and flow rate were studied. Unexpectedly, it was found that under some conditions, a higher buffer concentration tended to decrease the retention of sinigrin. Another competitive interaction might have taken place as the concentration of the ion-pair reagent increased. The mechanism that led to this phenomenon can be investigated in the future. Such a study may provide more insight of the dynamics inside the complicated column matrix. In all HPLC parameters tested, the pH of the aqueous buffer appeared to have more unpredictable effects on the retention of sinigrin as well as on column efficiency. For future recommendations, it is encouraged that the retention of the compound be investigated as a function of buffer strength at each pH value taking into account the variation of organic modifier.

As could be seen from Table B-7 (Appendix B), all the HPLC conditions tested had significant main effects on the retention of sinigrin (k) and many of them had interaction effects. Also, the difference of the two C18 columns tested was indicated in the chromatographic behavior of sinigrin during method development. In this study, a mathematical model was built for these two columns (separately and together) within each factorial matrix at various profiles using a standard regression model (Table B-8, Appendix B). Most of the HPLC variables were highly correlated to the retention of sinigrin, suggesting the regression model be linear. The strongest correlations were observed for column temperature, %acetonitrile as well as the sinigrin standard concentration when the two C18 columns were modeled separately. The combination of these three predictors along with the constant simulated accounted for over 95% of the variation of the k value of sinigrin. Further examination of the results showed that only the predictors of column temperature and %acetonitrile had a significant contribution to the prediction model for sinigrin, while the standardized regression weight of sinigrin standard concentration was not significant. Figure 4-2 shows simulations of sinigrin retention in the two C18 columns during the change of column temperature. Three levels of acetonitrile concentration were taken into account.

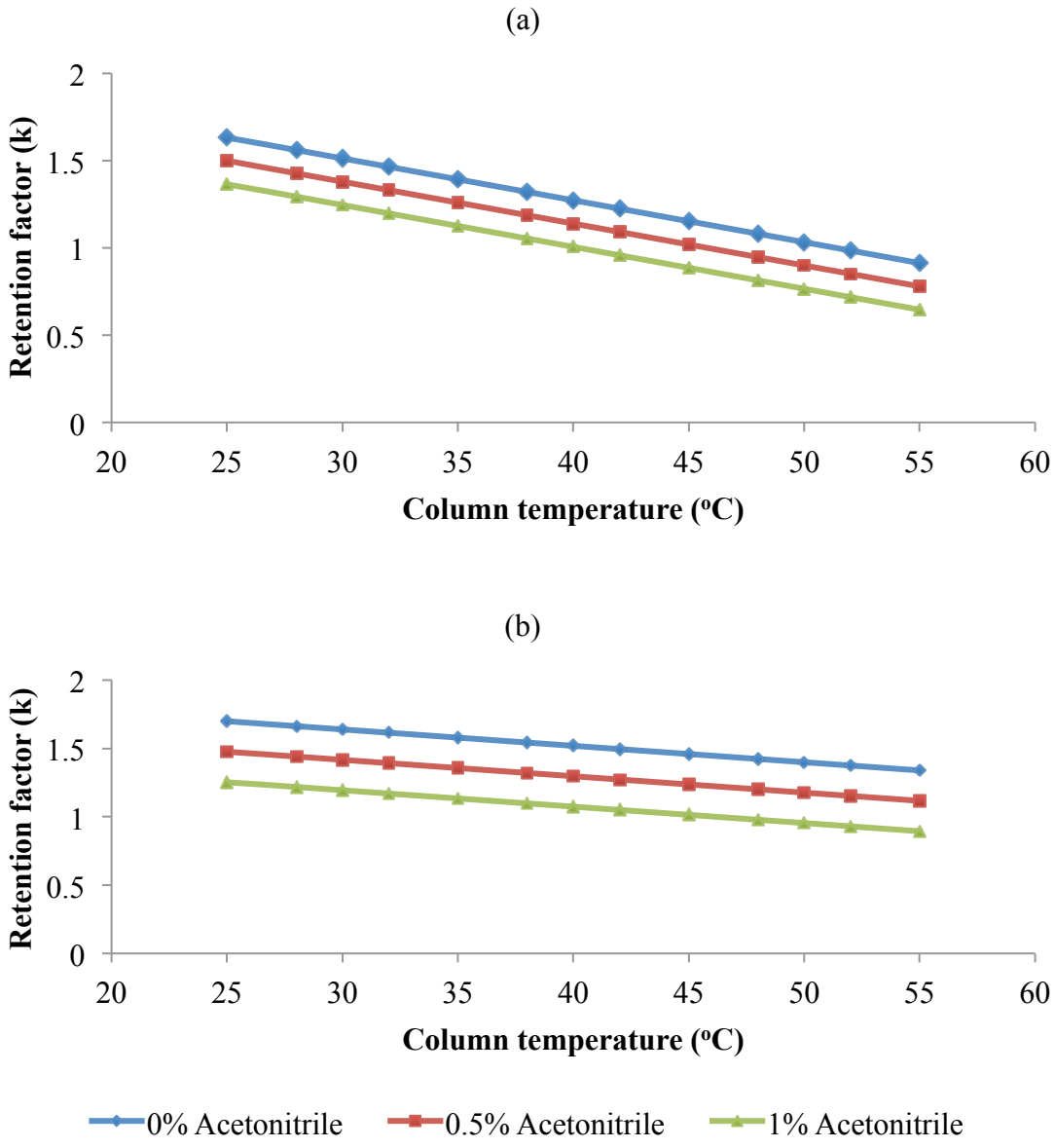


Figure 4-2 Simulation of sinigrin retention with different column temperatures set on (a) Eclipse C18 column, and (b) ODS-4 C18 column.

4.1.8 An attempt to use a monolithic column

Traditional LC columns are filled with particles while monolithic columns have a single porous silica rod or porous polymer (Unger *et al*., 2013). Having a much higher external porosity than particle packed columns, monolithic columns have the advantages of fast separation with short analysis time (Unger *et al*., 2013). In another test, an attempt was made to test the LC conditions on a reversed phase silica monolith column. Successful application of this column could further shorten the analysis time, while maintaining the compound retention. A Chromolith® reversed phase column as described in section 3.2 was used, with the temperature set at 25°C. An isocratic buffer of 100% of aqueous TMAB (10 mM, pH 5) was used for the elution of the compounds. Flow rates from 1 to 3.5 mL/min were studied. Good reproducibility of retention was achieved with the Chromolith® column, as indicated in the low value of CV% presented in Table B-9 (Appendix B). There was a slight increase in the retention of sinigrin in both matrices when the flow rate increased, and yet the highest *k* value (0.83) was lower than 1, which was considered inadequate for later separation between the analyte and other co-extract products from the more complex plant matrix. It was shown in a previous section (4.1.6) that changing the flow rate on the analytical column caused variation in the column efficiency, which was also supported by the results obtained in this section. The number of theoretical plates (*N*) calculated decreased by approximately 550, with an increase of flow rate every 0.5 mL/min.

Analysis of variance of the different factors on the retention of sinigrin is shown in Table B-10 (Appendix B). As could be seen from the table, preparation for the standards using water or mobile phase would not cause a significant effect on the retention ($p = 0.261$).

4.2 HPLC method validation for sinigrin

4.2.1 Pre-validation

As method pre-validation, sinigrin standard solutions were prepared both in water and mobile phase at the same concentrations (1-100 μg/mL), to validate that the HPLC mobile phase, as the matrix would not compromise the compound's retention and other chromatographic behaviors. As could be seen in Table C-1 (Appendix C), sinigrin standards that were prepared in water and the mobile phase behaved similarly to each other chromatographically. This also suggests that the optimized aqueous TMAB solution may present a high level of stability towards different matrices. Analysis of variance revealed that the C18 columns, the concentrations of

sinigrin standards as well as solvents used for preparing sinigrin standards all had a significant main effect ($p < 0.05$) on the retention (k) and detection (peak area) of sinigrin (Table C-2, Appendix C). Interaction effects between these factors were all significant ($p < 0.05$) on the detection, while the interaction between sinigrin standard concentration and matrix, and that between C18 columns used, sinigrin standard concentration and matrix were not significant on the retention of sinigrin. This also suggested that even for standards with relatively high purity, a change in the matrix would lead to deviation in the measurement of the analyte in HPLC. Good linearity (Figure 4-3) was achieved on all four combinations, with similar results within columns. The slope obtained with ODS-4 C18 column was greater than that with the Eclipse C18 column, indicating higher sensitivity for narrower peaks.

HPLC method development is a process where multiple variables are tested and adjusted to reach the optimal conditions for the measurement of analyte(s). The reliability of RP-HPLC depends on many factors, such as the mobile phase composition, addition of organic modifier, bonding material of the column, and the system temperature - many of which have been tested during the method development in current work. For the analysis of glucosinolate sinigrin using RP-HPLC, the main purpose is to achieve sufficient retention of the compound on the C18 columns for further quantitative analysis in complex materials. Both columns that were tested have proven the ability to achieve separative target along with good sensitivity and linearity. The ODS-4 C18 column was preferable as retention was more stable and column efficiency was greater. The ODS-4 C18 column was, therefore, used for the validation of the method following the directions of ICH (ICH, 2005).

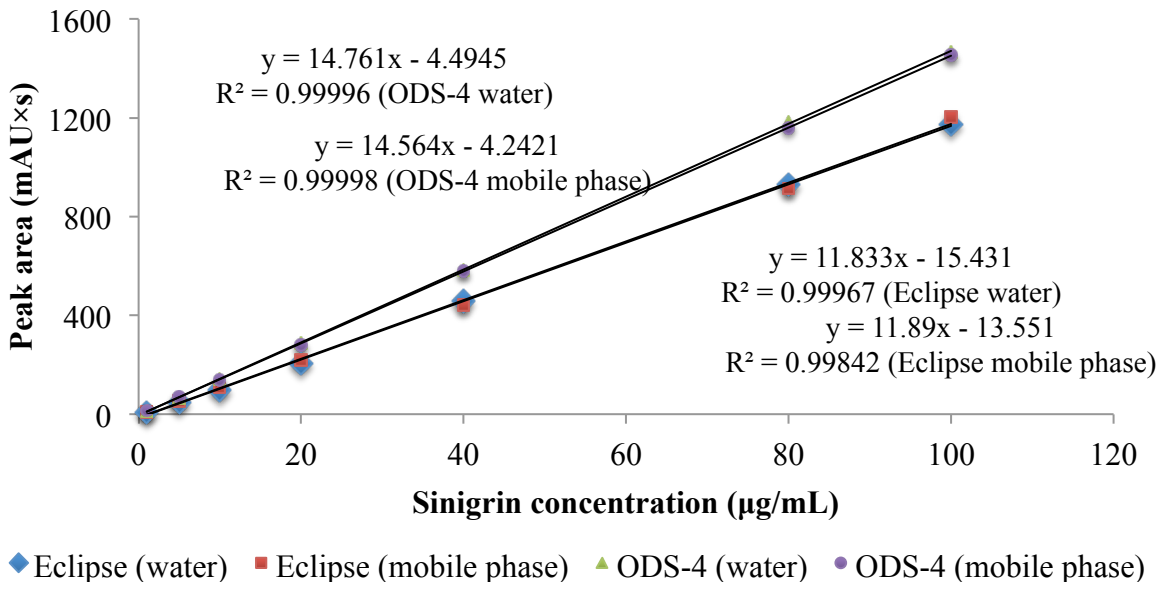


Figure 4-3 Calibration curves for sinigrin prepared in two solvents (water and mobile phase) on two columns. Range: 1-100 µg/mL.

4.2.2 Detection limit (DL) and quantitation limit (QL)

Sinigrin standards at eight concentrations from 0.1 to 5 µg/mL were diluted from the stock solution to determine the detection limit (DL) and quantitation limit (QL). As described in the ICH guideline (2005), there are three ways to determine DL and QL, amongst which the method based on the slope and standard deviation of the calibration curve was employed in current work to limit the possible discrepancy of results from the signal-to-noise ratio method, as the latter method requires manual measurement of the signals from the chromatograms. The range of standards for this study was determined based on 0-1% of the estimated amount of analyte (sinigrin) in the *B. carinata* seed materials. Mean value for peak areas against each concentration was calculated from four consecutive days of analyses (Table C-3, Appendix C). The largest coefficient of variation (CV%) resulted from the lowest concentration tested (0.1 µg/mL), yet the value (8.28%) was well below that determined by Vial and Jardy (1999), who suggested that a suitable QL could be determined as the concentration level, whose resultant peak area reached a predefined CV% of 12%. A linear regression analysis was performed, from which good correlation (R^2 = 0.99964) was achieved (Figure 4-4, a). It was worth noting that while the DL and QL were obtained based on the standard deviation and slope as described in

Equation 3.4 and 3.5, the ICH guideline (2005) provided two ways of estimating the standard deviation of the response, and specifically, two options within the category that was based on calibration curve, as is discussed here. In current work, the standard deviation of the regression line's intercept (σ) obtained was 0.096 and the residual standard deviation of the line ($s_{y/x}$) was 0.65, both of which could serve as the a measure of the standard deviation of the response. Therefore two values were obtained for both DL and QL. On the other hand, based on the result obtained from the present work that CV% of peak area as response of 0.1 µg/mL was already over 5%, it was determined that the DL and QL be set at 0.16 µg/mL and 0.48 µg/mL for accuracy according to the method. In addition, Vial and Jardy (1999) proposed another approach of estimating DL and QL, the method and results from which are also shown in Table C-4 (Appendix C).

4.2.3 Linearity

Sinigrin standard solutions at ten concentrations in the range from 10 to 1000 µg/mL were used to construct an external calibration curve from four consecutive days of analyses. Mean value for peak areas against each concentration was calculated from all the analyses (Table C-5, Appendix C). The standards were prepared in the mobile phase from the stock solution on each day. Good linearity was achieved on all four days, with the correlation coefficient $R^2 > 0.99999$ calculated by Microsoft Excel (Figure 4-4, b). The calibration curve constructed was used as the quantitation equation for calculating the concentration of quality control samples for recovery rates.

4.2.4 Accuracy and precision

The quality control samples representing five levels within the range of the calibration curves served as a means of investigating the accuracy and precision of the proposed HPLC method. Table C-6 (Appendix C) shows the retention times, concentration recovered, recovery rates, and the corresponding CVs% of the quality control samples from one day (repeatability) and four days (intermediate precision). The recovery rates were around 100%, except for the sample at 16 µg/mL (103-107%), which was also the lowest concentration tested. CVs% for retention times and recovery (%) were all within 1% within days, indicating good repeatability; the values were within 2% for recovery (%), and 3% for retention time as measures of intermediate precision.

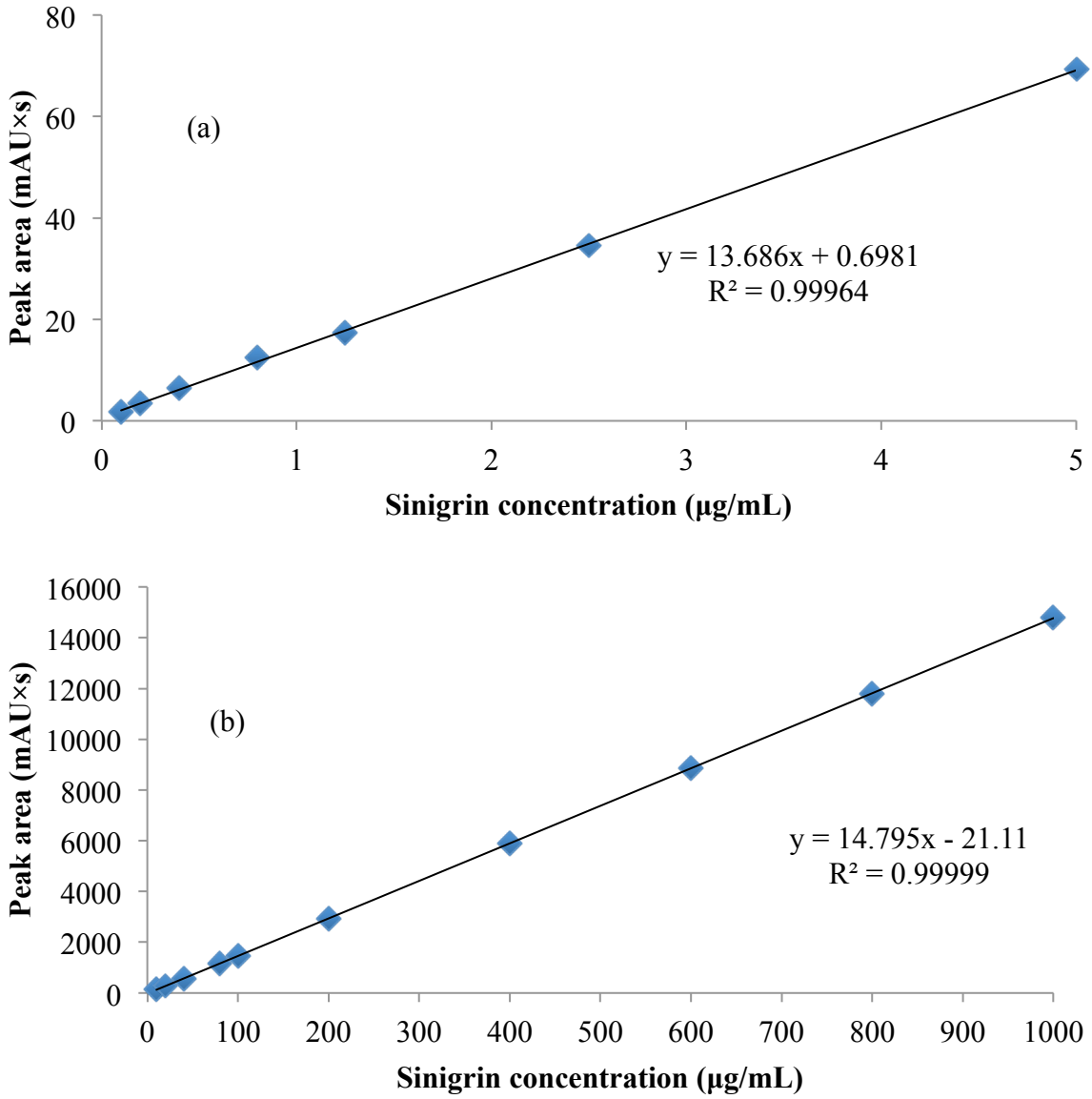


Figure 4-4 Calibration curves for sinigrin (in mobile phase). (a) 0.1-5 µg/mL and (b) 10-1000 µg/mL.

4.3 Sinigrin distribution in *Brassica carinata* seed

4.3.1 Crude oil yield from pre-pressing

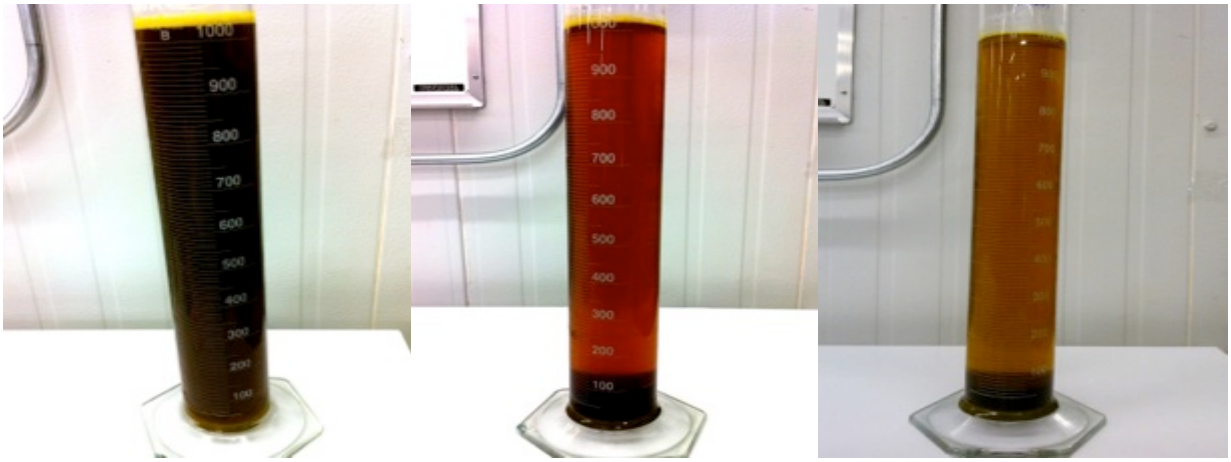
The *B. carinata* seed used in this study were of bulk standard, which contained a few impurities such as straw, stone and sand. An extra sieving step was performed prior to crude oil pre-pressing. Seed after sieving was more suitable for further processing (Figure 4-5).

Crude oil 309.2 (± 7.3) g and seed meal 681.2 (± 4.1) g were obtained after pressing 1000.0 g of *B. carinata* seed that was one-year-old. For four-year-old seed with the same amount, crude oil 260.9 (± 2.5) g and seed meal 737.2 (± 6.1) g were obtained. There was some weight loss for both the one-year-old (9.5 g) and four-year-old (1.9 g) seed, which may have been caused by some residues that remained in the expeller. The oil yield of *B. carinata* seed (30.9% and 26.1% for the one-year-old and four-year-old seed, respectively) obtained from pre-pressing was slightly lower than previous results on the same species (Getinet *et al.*, 1996), and this was most likely due to the different techniques for measuring the oil content. During pre-pressing, the seed meal carried a certain amount of oil (see section 4.3.2). A more accurate look at the oil content of the species would be through the conduct using Goldfisch (solvent) extraction. Oil solid residues (6.6 g) were produced from 3000.0 g of the one-year-old *B. carinata* seed (Figure 4-6). For the four-year-old *Brassica carinata* seed, 4.1 g of oil solid residues were accumulated from crude oil (250 mL).

(a) (b) (c)

Figure 4-5 *B. carinata* seed (1-year-old): (a) impurities stayed on 2.8 mm sieve; (b) impurities through 1.18 mm sieve; and (c) sieved seed.

(a) (b)



(c) (d) **(d)**

Figure 4-6 *B. carinata* seed processing products: (a) seed meal; (b) dried oil solid; (c) freshly pressed crude oil; (d) crude oil settled for 1 day; and (e) crude oil settled for 2 days.

(a) (b)

Figure 4-7 Sieved *B. carinata* seed: (a) 1-year-old and (b) 4-year-old.

4.3.2 Oil content of *Brassica carinata* seed (Goldfisch)

The oil content determined on dry samples using Goldfisch extraction (solvent extraction) is shown in Table 4-1. While it was measured on an "as is" basis, seed had higher oil content than the most of the accessions measured previously (Warwick *et al*., 2006). The variation of seed quality among traits could attribute to many factors, including genetics, environment, growing conditions and age (Hayward, 2012). In this case, the one-year-old yellow-seeded *B. carinata* had higher oil content than the four-year-oil brown-seeded *B. carinata* (Figure 4-7), and results obtained also agree with a previous study (Getinet *et al*., 1996).

In a research paper on Brassicaceae oil, Ratanapariyanuch *et al*. (2013) demonstrated the linear relationship between oil content and oil yield of several Brassicaceae species and in current work, oil content and oil yield of the *B. carinata* seed tested also presented a similar general linear relationship as shown in Equation 4.2, with $R^2 > 0.995$.

$$y = 0.9348x - 6.2735 \quad (4.2)$$

Where y refers to the oil yield of *B. carinata* seed obtained from pressing as described in 4.3.1, and x refers to the oil content obtained from Goldfisch extraction.

Table 4-1 Oil content of *Brassica carinata* seed and seed meal (as is basis, n=3)

Yellow-seeded (1-yr-old)		
Whole seed[a]	Mean	39.49
	SD	0.97
Seed meal	Mean	17.32
	SD	0.15
Brown-seeded (4-yr-old)		
Whole seed	Mean	34.63
	SD	0.04
Seed meal	Mean	17.79
	SD	0.36

[a] One outlier removed.

4.3.3 Sinigrin distribution during primary processing of *Brassica carinata* seed

4.3.3.1 Extraction study

For the extraction of sinigrin from mustard seed, Cools and Terry (2012) concluded that boiling 50% (v/v) aq. acetonitrile was the most efficient solvent amongst the four they tested. Conventionally, 70% (v/v) aq. methanol was more often chosen as the extraction solvent. In current work, these two solvents were compared for their efficiency of extracting sinigrin from *B. carinata* whole seed. Unlike previous studies, neither of them was heated in order to prevent thermal degradation, and the whole sample preparation step was performed at ambient temperature in current work. *B. carinata* seed was ground, weighed (50 mg) and extracted with the solvent (1.5 mL) as described above in the section 3. The resulting solution was mixed and centrifuged, and the supernatant obtained was collected while the pellet was re-extracted with the solvent (1.5 mL). This extraction was repeated until no sinigrin was detected from the extract, as was indicated in the ESI-MS detector (negative mode). HPLC results were in agreement with those obtained from MS infusion for both solvents: sinigrin was hardly detected during the eighth time of extraction using 70% (v/v) aq. methanol as well as during the fourth time of extraction using 50% (v/v) aq. acetonitrile (Table 4-2).

With peak areas of sinigrin being the indicator of extraction efficiency, it was noted that 50% (v/v) aq. acetonitrile was able to extract more sinigrin during the first extraction, while 70% (v/v) aq. methanol was able to extract much more of the compound totally. The fact that very little sinigrin was detected in the extract after the third time of extraction was not sufficiently convincing to determine that the extraction was complete. Rather, it may instead suggest that the solvent used was not strong enough to extract as much analyte in the complex matrix. This assumption was supported by the large difference of sums of peak areas from different times of extraction between 50% (v/v) aq. acetonitrile (16033.87) and 70% (v/v) aq. methanol (22043.50). 70% (v/v) aq. methanol was therefore chosen as the solvent for extraction of sinigrin in the following work. It is worth noting that researchers who previous recommended the use of 50% (v/v) aq. acetonitrile over others conducted the extraction with boiling solvent (Cools and Terry, 2012; Tsao *et al*., 2002), while in this study, plastic centrifuge tubes were employed instead of glass vials during extraction, in order to stay consistent with later studies where a

larger amount of samples were to be tested, an experiment conducted at room temperature was preferred and chosen.

Table 4-2 Efficiency of two solvents on repeated extractions of sinigrin

Times of extraction (1.5 mL per time)	(n^a=3)	t_R (min)	Peak area (mAU × s)
		70% (v/v) aq. methanol	
1	Mean	5.58	11752.70
	CV (%)	0.44	7.08
2	Mean	5.66	5437.00
	CV (%)	0.09	2.31
3	Mean	5.73	2129.93
	CV (%)	0.12	11.55
4	Mean	5.73	1466.20
	CV (%)	0.24	16.28
5	Mean	5.71	798.13
	CV (%)	0.13	20.22
6	Mean	5.81	455.33
	CV (%)	0.20	28.53
7	Mean	5.84	4.20
	CV (%)	0.33	48.33
		50% (v/v) aq. acetonitrile	
1	Mean	5.51	14563.50
	CV (%)	0.18	2.30
2	Mean	5.74	1417.73
	CV (%)	0.21	3.21
3	Mean	5.77	52.63
	CV (%)	0.14	40.51

[a] Triplicate extraction

4.3.3.2 Measurements of sinigrin in seed fractions with standard addition method

During the processing of *Brassica carinata*, seed meal, oil and suspended oil solids were produced (Figure 4-6). It was the purpose of this project to measure the level of sinigrin in the whole seed as well as in these products. Being one of the major components in the species, sinigrin was well separated from other components in the extract as indicated by the stable retention times (Table 4-3). Quantitation using the method of standard addition helped to identify the analyte sinigrin in the complex matrix, assuming that with an increase in the amount of standards added, area of the UV peak appeared at the same retention time would increase (Figure 4-8). A calibration curve was constructed (Figure 4-9) with unspiked and spiked (six points) samples for each material and a linear equation (in the form of $y = mx + b$) was obtained using the least square method. The concentration of sinigrin in the unspiked sample (x in the equation) was calculated as the x-intercept in the equation (when y was set to 0). It is worth noting that when the sinigrin standards (0, 100, 200, 400, 600, and 800 μg/mL) were spiked into the sample concentrates, their eventual concentrations in the re-constituted samples would change as a result of the change in final volume, and it was these final concentrations (0, 25, 50, 100, 150, and 200 μg/mL) that were used to construct the calibration curve. Sinigrin level in *B. carinata* whole seed obtained was 29.09 (± 0.56) μg/mg on an "as is" basis (Table 4-3), which was lower than that measured by Bellostas *et al*. (2007). In their paper, total glucosinolate constituted 116 μmol/g ripe seed of *B. carinata* on a dry basis, with more then 900 mmol/mol sinigrin, which was about 41.49 μg/mg (Bellostas *et al*., 2007).

Compared to other *Brassica* species, the sinigrin concentration obtained in current work was also slightly lower than that recently measured in *B. juncea* (L.), which was 32 mg/g seed (Cools and Terry, 2012). In determining the sinigrin concentration in a collection of *Brassica* seeds, Rangkadilok *et al*. (2002) reported the great variation in seed sinigrin concentrations between and within species, in which the highest and lowest level of sinigrin (55.03 μmol/g and 0.05 μmol/g measured in dry weight) was found in different genotypes of *B. nigra.* The pronounced difference between the sinigrin level obtained in this study and in other literatures is likely due to many factors. In addition to variation arising from the species and growing

environment of seed, the extraction protocol, measurement equipment as well as method of quantitation may also contribute to the measured value of glucosinolate. Specifically, Cools and Terry (2012) estimated the compound's concentration in seed against an external calibration curve built by standard solutions using HPLC-DAD, while Rangkadilok *et al*. (2002) spiked standards to the samples before extraction to calculate the recovery. In the research report described by Bellostas *et al*. (2007) mentioned above, the authors spiked other types of glucosinolate as internal standards before extraction, and the analysis was performed on MECC. In the experience of the author, sample matrix is prone to significantly influence the detection and quantitation of glucosinolates, not only in MS analysis through the deviate ionization, but also in other analytical techniques such as HPLC-DAD, which was discussed in the next section (4.3.3.3) as concluded as matrix effect. It was therefore emphasized during the current project that quantitation of glucosinolates was conducted using the method of standard addition, or internal standards when authentic samples were not available at the time of experiments. And this may explain partially the difference in sinigrin content obtained in current work and previous literature.

Seed meal is the most important by-product from oilseed processing and it contains the highest level of sinigrin (46.72 ± 0.92 μg/mg) among the *B. carinata* materials tested from current work. It might suggest that this material may not be the best candidate as feed for farm animals (Alexander *et al*., 2008; Snowdon *et al*., 2007). The sinigrin level in *B. carinata* crude oil (0.075 ± 0.0048 μg/mg) was calculated from the yield of crude oil extract (extraction step described in section 3.3.3**)**. Eventually 80 mg of extract concentrate was produced from one replicate of crude oil, and the yield, therefore, was 0.16%. The appearance of dried crude oil extract was similar to that of the oil suspended solids. Considering the processing procedures of oilseed, the crude oil extract, oil suspended solids and seed meal could be categorized roughly into one group with similar components. The similar sinigrin level in seed meal (46.72 ± 0.92 μg/mg) and the crude oil extract (46.97 ± 2.98 μg/mg) supported this assumption. Oil content of the *B. carinata* whole seed and meal (one-year-old) was 39.49% (± 0.97) and 17.32% (± 0.15) respectively, as shown in section 4.3.2. Sinigrin levels in the defatted seed and defatted seed meal were estimated to be 48.04 (± 0.77) μg/mg (48.09 ± 0.77 μg/mg if the sinigrin level in crude oil was omitted) and 56.49 (± 0.10) μg/mg (56.51 ± 0.10 μg/mg if the sinigrin level in crude oil was omitted), respectively.

Table 4-3 Sinigrin distribution during primary processing of *Brassica carinata*

Material		t_R[a] (min)	Quantitation equation[b]	R squared	Sinigrin level (µg/mg)
Whole seed	Mean	5.63	y=15.576x+5889.3	0.9972	29.09
	SD	0.07			0.56
Seed meal	Mean	5.56	y=16.302x+9708.5	0.9189	46.72
	SD	0.02			0.92
Crude oil extract	Mean	5.65	y=14.461x+693.23	0.9996	46.97
	SD	0.04			2.98
Crude oil[c]	Mean				0.075
	SD				0.00

[a] Mean value of retention times (t_R) for eighteen runs.

[b] y refers to the peak area for sinigrin generated by detector against x, which refers to the concentration of spiked sinigrin in the re-constituted sample.

[c] Sinigrin level in crude oil was determined by the crude oil extract yield (n=1) and the compound's level in crude oil extract.

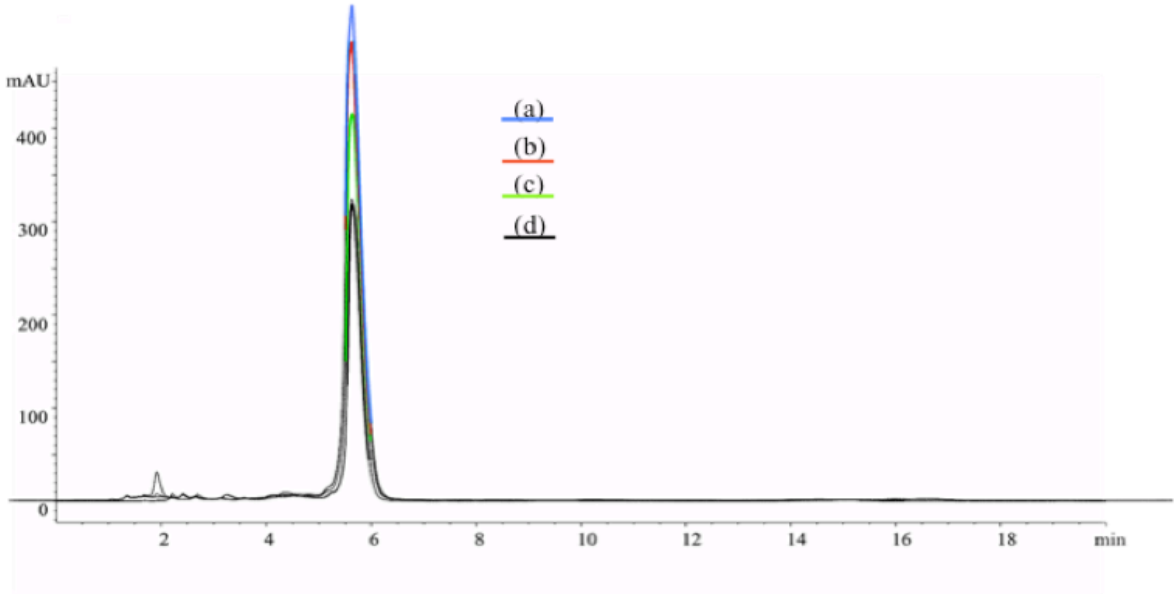


Figure 4-8 The overlapping HPLC chromatograms of *B. carinata* seed extracts spiked and unspiked with sinigrin standards. (a), (b), (c): samples spiked with 800, 600 and

200 µg/mL sinigrin standard, respectively; and (d) sample spiked with no sinigrin standard.

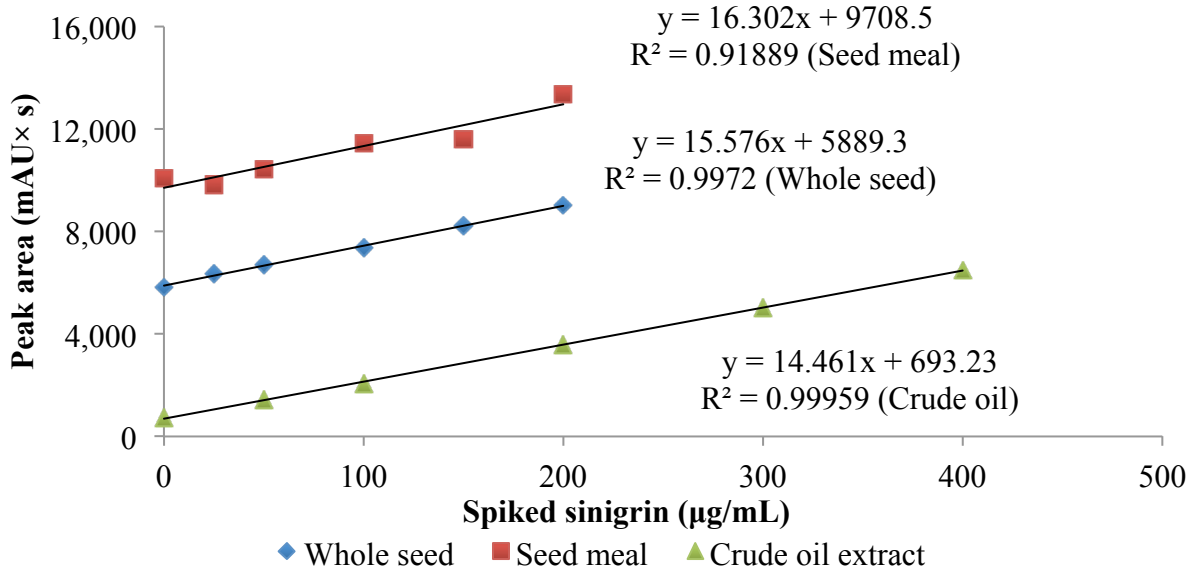


Figure 4-9 Calibration curves for *B. carinata* samples using the standard addition method. Concentrations of sinigrin in the unspiked samples were the positive x values calculated by setting y to 0 in the equation.

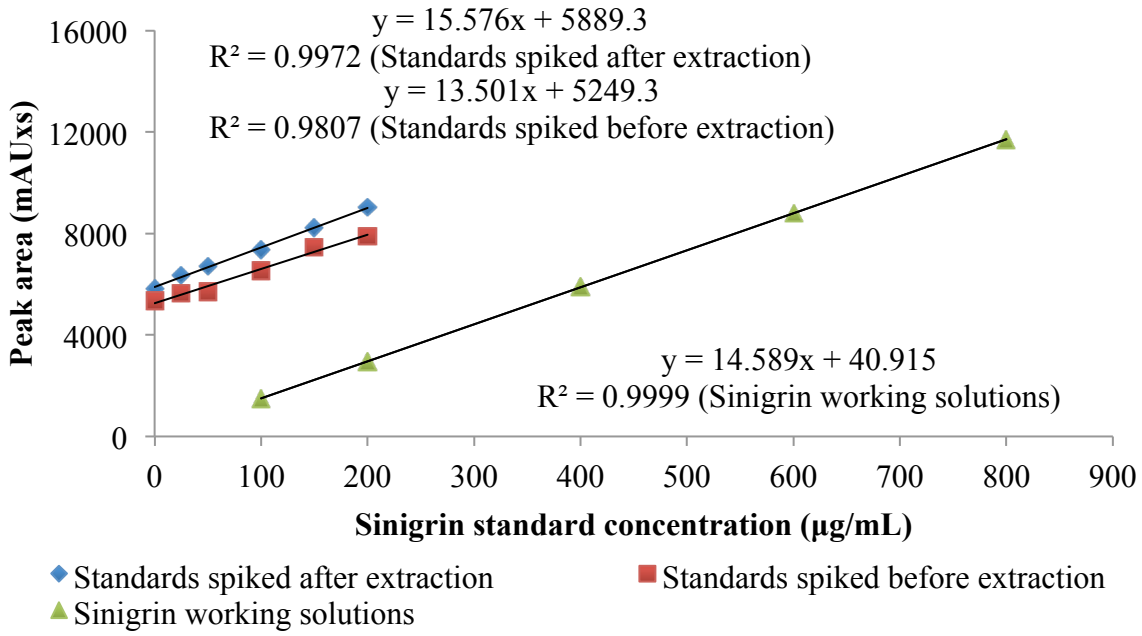


Figure 4-10 Calibration curves for determination of sinigrin using three methods.

4.3.3.3 Further discussion about matrix effect

In further determining the matrix effect and recovery rate of the proposed protocol for sinigrin measurement in *B. carinata* seed materials, three methods of quantitation were used and compared. Specifically, sinigrin standards in the range from 100 to 800 μg/mL were used to construct an external calibration curve. These standards were also used to spike into the *B. carinata* ground seed, either before or after extraction. The sinigrin level in *B. carinata* seed was determined in these three ways, and the results were compared (Table 4-4 and Figure 4-10). The concentrations obtained were similar to each other, which could be attributed to the stable performance of the system and the extraction procedure. It is worth mentioning again that the x-axis represents the standards' concentrations in the re-constituted sample that was injected into the system, not the original concentration of the standards used to spike.

Assessment of matrix effect of the proposed HPLC method was referenced from a research of Matuszewski *et al*. (2003) with modifications based on practical situation. For calculations of the matrix effect, the sinigrin level in the unspiked samples in the first set of samples was calculated (30.55 μg/mL) from the external calibration curve of the third set of samples (neat solutions) (Table 4-4); the value was used as the default concentration, which was then added to the spiked standards' concentrations to calculate the theoretical peak areas. In determining the process efficiency, the sinigrin level in the unspiked samples in the second set of samples was calculated (28.75 μg/mL) from the external calibration curve of the third set of samples (neat solutions); the value was then added to the spiked standards' concentrations to calculate the theoretical peak areas. The overall matrix effect (%) obtained was 104.44% (Table 4-5). This reveals that matrix effect took place during analysis of sinigrin using the HPLC method, which was caused by other components in the *B. carinata* seed matrix, and it resulted in overestimation of the sinigrin level in the samples. While the absolute matrix effect was observed, it is believed that relative matrix effect may also occur - matrix effects between different seed fractions from the same plot (origin), between the same kinds of fractions from different plots, and between analysis using different detectors are expected to vary. In current work, the matrix effect for one analyte in one type of sample from the same origin using an

HPLC method was assessed, and results obtained therefore revealed the presence of matrix effect as well as the extent and direction that this effect had on the measurement within the range as described.

Table 4-4 Sinigrin level in *B. carinata* seed determined by three methods

Method		t_R[a] (min)	Quantitation equation[b]	R squared	Sinigrin level (μg/mg)
Spiked after extraction	Mean	5.63	y=15.576x+5889.3	0.9972	29.09
	SD	0.07			0.56
Spiked before	Mean	5.65	y=13.501x+5249.3	0.9807	30.71
	SD	0.05			0.94
External calibration	Mean	5.65	y=14.589x+40.915	0.9999	30.55/28.75
	SD	0.06			0.32/0.41

[a] Mean value of retention time for eighteen runs

[b] y = peak area generated by detector against x; x = concentration of sinigrin standards in the sample injected

Table 4-5 Matrix effect, recovery rate and processing efficiency for measurement of sinigrin in *B. carinata* seed

Nominal conc. (μg/mL)		Matrix effect (%)	Recovery rate (%)	Processing efficiency (%)
0	Mean	100.00	91.75	100.00
	CV (%)	1.03	3.14	1.41
100	Mean	105.64	89.13	97.52
	CV (%)	2.45	10.60	5.45
200	Mean	107.08	84.98	95.41
	CV (%)	0.14	1.30	1.44
400	Mean	103.47	88.87	96.82
	CV (%)	4.42	5.10	0.16
600	Mean	105.46	90.76	97.16
	CV (%)	2.86	1.38	0.04
800	Mean	104.98	87.38	95.82
	CV (%)	2.11	0.90	0.73
	Overall mean	104.44	88.81	97.12
	CV (%)	3.09	4.97	2.56

The range of CVs (%) of actual peak areas were 0.98-5.22% and 0.044-5.88% for samples with standards spiked after and before extractions, respectively. The latter one was used to evaluate the recovery of analyte from the extraction procedure, and the comparable values obtained suggest that the effect of variation of recovery may be minor. Quite often, recovery rate (%) is a variable to be evaluated in a proposed method, in which standards are added to the samples before extraction. In current work, the processing efficiency (%) was more indicative of the recovery from the extraction step alone while the calculation of the actual recovery rate (%) also took into account the apparent presence of matrix effect on quantitative measurement, and therefore was more reflective of the overall recovery of the proposed protocol, i.e., from sample preparation to analytical measurement. Yet, an even more comprehensive examination will be to conduct the analysis of *B. carinata* samples from more origins, at least five as was suggested by Matuszewski *et al*. (2003).

To minimize matrix effect on the analysis of analyte (sinigrin) in plant materials, efforts should be put on the optimization of the extraction procedure that isolates sinigrin from other interfering components from the matrix. For the chromatographic condition or any other analytical techniques, there is always space for improvement.

4.4 Detection and identification of glucosinolates in *Camelina sativa*

The species *Camelina sative* produces three major glucosinolates that differ in the length of the n-alkyl portion for each compound (indicated in the abbreviated names as GS9, GS10 and GS11). It is likely that these three compounds have similar chromatographic behaviors. During a preliminary experiment, the performance of two sets of composition as the mobile phase for HPLC-MS analysis were compared: a gradient of H_2O with 0.1% formic acid and acetonitrile with 0.1% formic acid and; and a gradient of H_2O with 0.25% acetic acid and methanol, the latter one of which were used by Berhow *et al.* (2013) at a ratio of 60%/40% (v/v) as starting gradient in HPLC-MS. Other HPLC conditions and MS parameters were held constant. Results showed that these compounds were readily detected in the *C. sativa* extract while the use of the first solvent composition gave a much better chromatographic resolution.

4.4.1 Detection of glucosinolates in crude oil of *Camelina sativa*

A preliminary experiment was conducted directly on freshly cold-pressed *C. sativa* oil (5 mL), which was mixed with 80% (v/v) aq. ethanol (1.25 mL). The crude extract was centrifuged, the supernatant filtered and injected to ESI-HPLC-MS system. While APCI was available, previous experience suggested that ESI source was more preferable for analytes with high polarity like glucosinolates. Further MS/MS (parent ions scan) analysis indicated the occurrence of three glucosinolates in the oil extract, from which ions with m/z 506, 520, and 534 were scanned that corresponded to the theoretical molecular weight of deprotonated GS9, GS10 and GS11. The three compounds were detected as they eluted at 4.8, 5.3, and 6.0 min as shown in Figure 4-11. This experiment not only detected *C. sativa* glucosinolates, but also suggested a relatively high concentration of these compounds in the freshly pressed crude oil, given the high signal intensity from only a small amount of material.

4.4.2 Detection of glucosinolates in camelina oil foot (dry oil solids)

During oil pressing, some solids were released and collected together with the oil. An assumption was made – based on the experiment described in section 4.4.1 as well as previous studies (Matthaus and Zubr, 2000) - that these solids would be enriched with glucosinolates. To validate this assumption, a pail of pressed *C. sativa* crude oil was settled for 2 days to allow these solids to settle to the bottom, resulting in a layer of black residue. This black residue, also known as oil solids, was collected along with some residual oil. While this mixture might still contained triglycerides, diglycerides, monoglycerides, sterols, and tocopherols (Aakman, 1983), most of the nonpolar lipids could be eluted from solids by hexane (polarity index: 0.1), and the polar fractions containing glucosinolates would remain.

The processed oil solid (Figure 4-12) was extracted and subject to the same LC-MS-ESI analysis as the *C. sativa* oil extract above. Signals with high intensities were detected for all three *C. sativa* glucosinolates (GS9, GS10 and GS11), which confirmed the assumption that the *C. sativa* oil solids contained high concentrations of glucosinolates.

In addition, a more specific MS/MS condition was developed, in order to further confirm the compounds' identities, as well as to investigate their MS fragmentation behavior for structure elucidation. Instead of auto MS/MS fragmentation, MS/MS analysis with multiple reactions monitoring (MRM) was used. Collision energy for each of the glucosinolates was set within a

range of 18 to 30 eV. At 18 eV, the energy was too low, and the glucosinolate remained almost intact without fragmentation. On the other hand, collision energy of 30 eV was too high, and the precursor ion was barely detectable. Eventually, collision energy of 21 eV was found to be suitable for glucosinolate with m/z 506, and collision energy of 22 eV was found to be suitable for glucosinolates with m/z 520 and 534. GS9, GS10 and GS11were detected at 4.9 min, 5.5 min, and 6.2 min, respectively, from the eluent and their corresponding MS fragmentation spectra were shown in Figure 4-13.

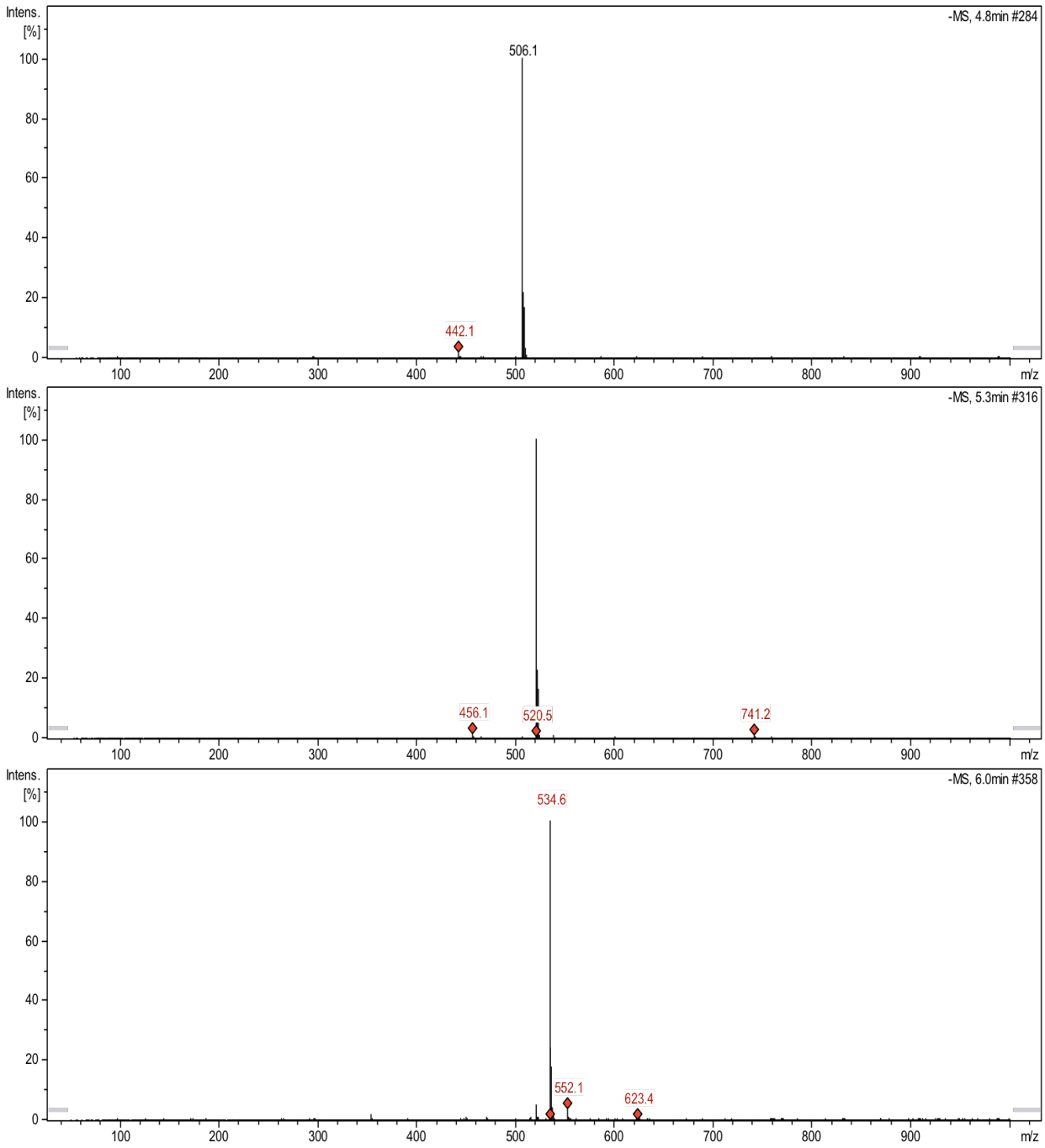
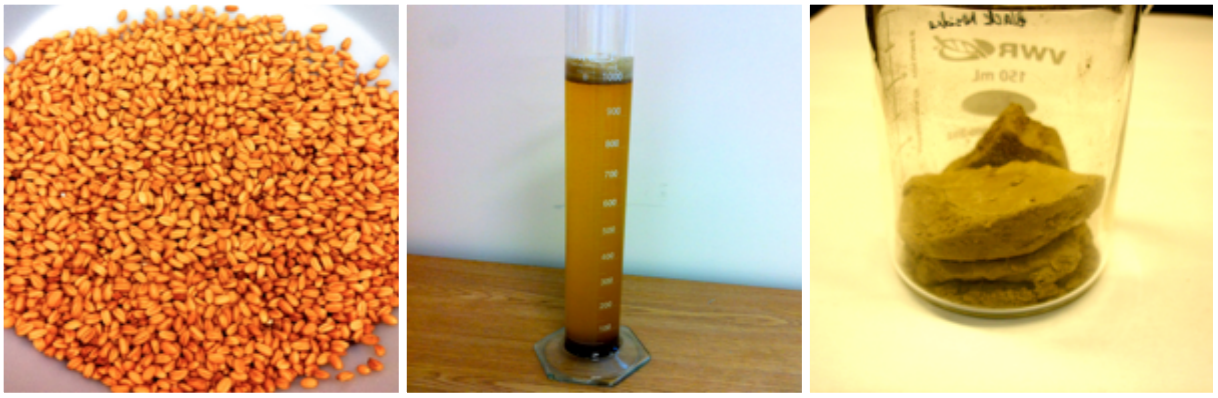


Figure 4-11 MS spectra of glucosinolates compounds ions detected in a sample of freshly pressed *C. sativa* oil extract.



(a) (b) (c)

Figure 4-12 *C. sativa* seed and products: (a) whole seed; (b) freshly pressed crude oil; and (c) dry processed oil solids.

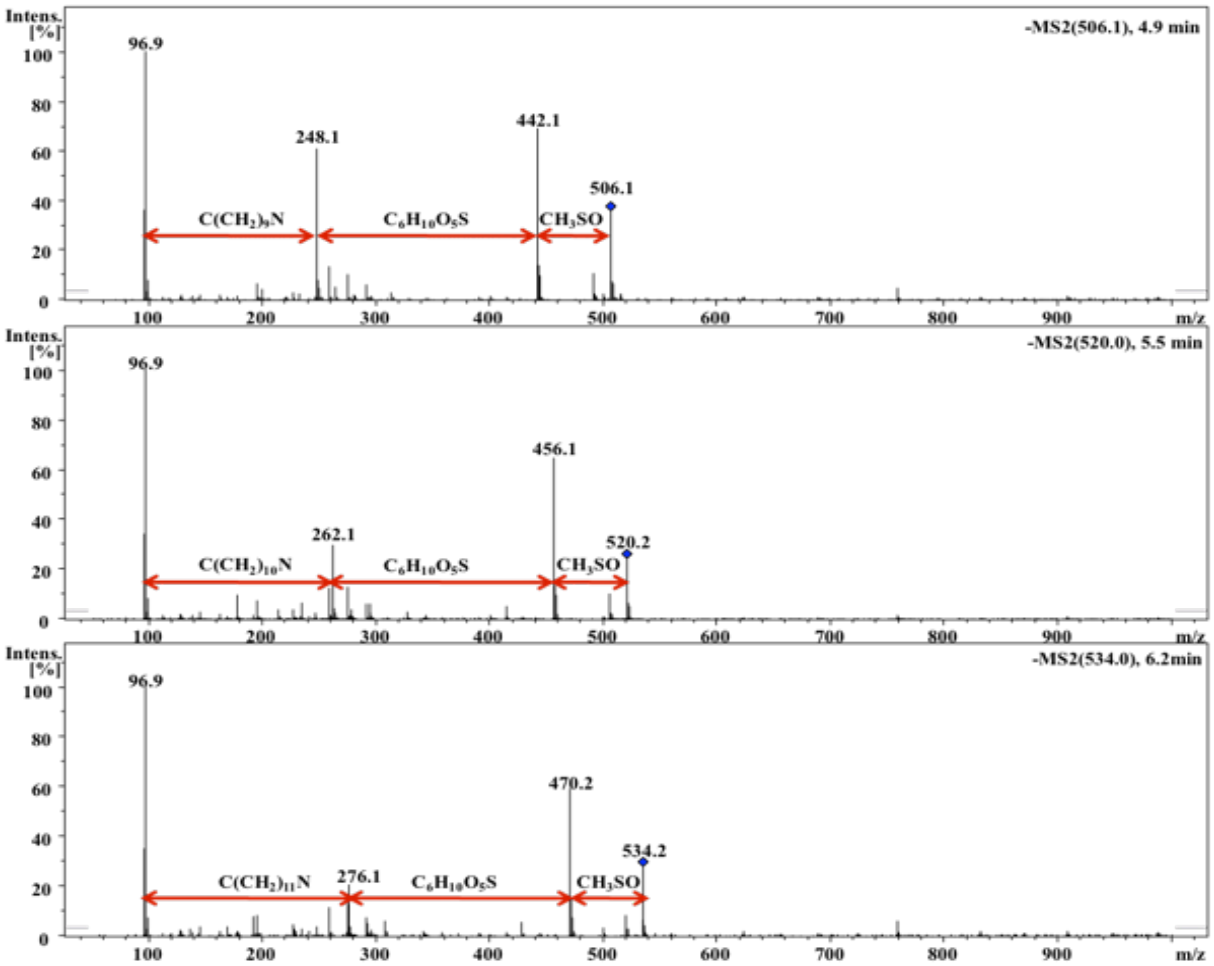


Figure 4-13 Fragment MS/MS spectra of glucosinolates with parent ions at m/z 506, 520, and 534 from the dry oil solid extract.

The MS/MS spectra of *C. sativa* glucosinolates show similar and consistent fragmentation behavior, revealing comparable ion dissociation reaction in the ESI-TOF mass spectrometer. Four product ions were detected in all three MS/MS fragmentation spectra with high intensities. The m/z 97 ion had the highest relative abundance in all three mass spectra, which was characteristic of the hydrogen sulfate $[HSO_4]^-$ ion. This is in agreement with reference work that applied other kinds of mass detectors to identify glucosionlates (Bennett *et al*., 2003; Cai *et al*., 2004; Mellon *et al*., 2002). The abundance of $[HSO_4]^-$ ion could also be used as the diagnostic ion for screening analysis, and as fragment ion for quantitation (Song *et al*., 2005). The second most intense product ion was at m/z 442 for GS9, 456 for GS10, and 470 for GS11, all of which could be traced back to the loss of the group $[CH_3SO]^-$ from the side chain. The neutral loss of the thioglucose group $[C_6H_{10}O_5S]^-$ remained constant, which resulted in m/z at 248 for GS9, 262 for GS10, and 276 for GS11. Other specific product ions with comparatively low abundance have also been detected, including m/z at 178, 195, 214, 259, 275, and 294.

This optimized LC-MS-ESI (MRM) method proved useful to identify and characterize the intact *C. sativa* glucosinolates with high selectivity and sensitivity, which would serve as a tool for screening the occurrence of the compounds in plant fractions as well as to examine the purity of later prepared standards.

4.4.3 A general HPLC-ESI-MS method to identify other components

While the specific MS/MS (MRM) analysis had its special uses as described above, a more general method could be used to identify other possible components present in the plant extract, with the same LC gradient program above using a Chromolith® Performance RP 18-e column. The retention times of the three glucosinolates using this column (6.3, 6.8 and 7.5 min for GS9, GS10, and GS11, respectively) were slightly longer than those measured using the FastGradient column (4.9, 5.5, and 6.2 min for GS9, GS10, and GS11, respectively), but had the same elution order. The chromatogram suggested once again that the re-constituted sample was highly enriched with *C. sativa* glucosinolates. Moreover, based on the similar MS/MS fragmentation behavior of these glucosinolates that are of similar structures to each other, assumptions were made that these glucosinolates would have the same reaction towards ionization in the ESI surface, which made the quantitation of them by measuring the extracted

ion signals likely (Figure 4-14**)**. Other components detected by mass spectrometer included compounds with m/z 209, 253, 283, 297, 311, 329, 343, and 345. The identities of these components were yet to be confirmed.

The LC-MS/MS methods developed to identify glucosinolates in *C. sativa* seed materials prove high specificity and selectivity. The MS/MS analysis prevents the interference from other co-eluting components that might be confusing in HPLC-DAD analysis, and can be used under various investigations for identification purpose. The other LC-MS method thereafter derived can be applied in more general screening; given highly similar structure of these three *C. sativa* glucosinolates, quantitation measurement can be realized using extracted ion chromatogram. The HPLC-MS conditions described here for detecting *C. sativa* glucosinolates was also presented by the author and co-workers of the current work in the CSBE 2013 Annual Conference (Yuan *et al*, 2013).

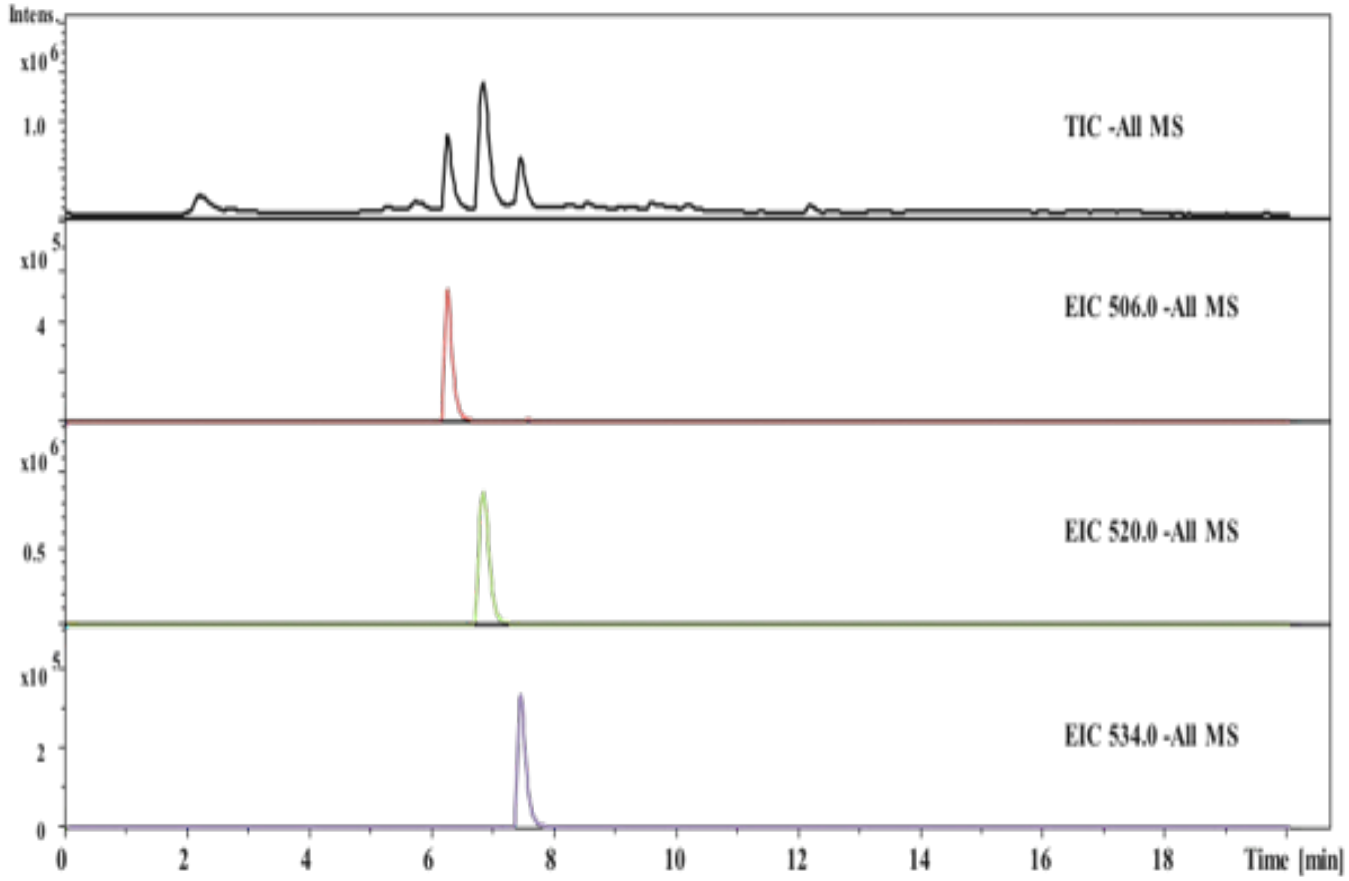


Figure 4-14 Total ion chromatogram and the extracted ion chromatograms of m/z 506, 520, and 534 of the re-constituted sample of the oil solid extract.

4.5 Glucosinolates isolation using semi-preparative and preparative HPLC

Currently none of the *C. sativa* glucosinolates are available for sale as standards for biological research. On the other hand, their structurally similar counterpart, glucoraphanin, has been available in the market for research as a potential nutritional supplement. The isolation and purification of glucoraphanin from broccoli seed using preparative HPLC or HSCCC has been reported (Fahey *et al*., 2003; Rochfort *et al.*, 2006). The technique of preparative HPLC was also applied to the current study for isolation. *C. sativa* fractions rich in glucosinolates are therefore needed as the raw material for preparing standards. While defatted seed meal recovered from oil extraction has been reported previously to be the part of the plant that contains considerable amounts of the corresponding glucosinolates for isolation (Berhow *et al*., 2013; Matthaus and Zubr, 2000), the processed oil foot (dry oil solid) has been shown to be a good candidate for preparing the compounds, as described in section 4.4.

Many of the studies that involve extraction of glucosinolates from plant fractions work for analytical measurement. While the use of 70% (v/v) aq. methanol was used more often (Clarke, 2010), ethanol (polarity index: 5.2) could be a suitable solvent for extracting glucosinolates from gum and other sugar derivatives. Van Megen (1983) reported that 75% (v/v) aq. ethanol was able to remove more than 99.5% glucosinolates from defatted rapeseed meal. A similar study was conducted by Finnigan and Lewis (1988), who examined the aqueous ethanol in the range of 0 - 100% (v/v), in order to extract glucosinolates from rapeseed meal, and noted that 80% (v/v) aq. ethanol was the most appropriate concentration, as it achieved proper extraction of the compounds while maintaining other components in the residue material. And this conclusion suits the need for glucosinolate extraction and isolation in current investigation. More recently, Campos *et al.* (2013) determined through surface response analysis that 60% (v/v) aq. ethanol to be the most efficient to extract glucosinolates from maca flour. According to their conclusion, the concentration of ethanol had a significant effect on the extraction efficiency of glucosinolates, the level of which was influenced by the type of the glucosinolates and plant species (Campos *et al.*, 2013). All three *C. sativa* glucosinolates were in the same chemical class as summarized by Fahey *et al.* (2001): they all have sulfur-containing aliphatic chains connected to the terminal methylsulfinyl groups. These would all contribute to consistent behavior in response to various extraction conditions. 80% (v/v) aq. ethanol was chosen to be the solvent for extraction of *C. sativa* fractions considering the experimental integrity, efficiency as well as the

characteristics of *C. sativa* seed.

4.5.1 Glucosinolates isolation using semi-preparative HPLC

Semi-preparative scale LC connected to a UV detector was used to isolate individual *C. sativa* glucosinolates from the oil solid extract. While 1 μL was enough for the mass spectrometer to detect these compounds, a much larger injection volume as well as a higher flow rate would be required for preparative work. The use of a Chromolith® SemiPrep RP-18e column was able to achieve a flow rate at 3.7 mL/min with 96 μL for each injection, while reasonable resolution and reproducibility were maintained for a minimum of ten injections. This semi-prep isolation method was developed from the analytical HPLC-MS method. The components of the mobile phase (H_2O, acetonitrile, formic acid water solution) allowed for a comparable transfer of the methods for scaling-up, and also simplified the later concentration step due to the solvents' reasonably high volatility. Through confirmation in HPLC-MS analysis, glucosinolates with m/z 506, 520, and 534 were detected separately in four fractions, which eluted continuously from 4.3 to 6.1 min (Figure 4-15). On the other hand, since the detection of these compounds was dependent on other co-eluting compounds that have high UV absorbance, these fractions also contained impurities. Fraction 2, collected from 4.7 to 5.3 min, was also found to contain compounds with m/z 294 and 741. Fraction 3, collected from 5.3 to 5.6 min, was also found to contain compound with m/z 609. Fraction 4, collected from 5.6 to 6.1 min was also found to contain compound with m/z 623.

This semi-preparative method was fast (10 min) and was able to isolate glucosinolates from the plant extract. Combined HPLC-MS analysis and this semi-preparative work proved conclusively that these three *C. sativa* glucosinolates had weak chromophores and were not readily detected with the UV detector, but they co-eluted with other components in the extract with stronger chromophores. This semi-preparative HPLC technique for isolating *C. sativa* glucosinolates was also presented by the author and co-workers of the current work in the CSBE 2013 Annual Conference (Yuan *et al.*, 2013).

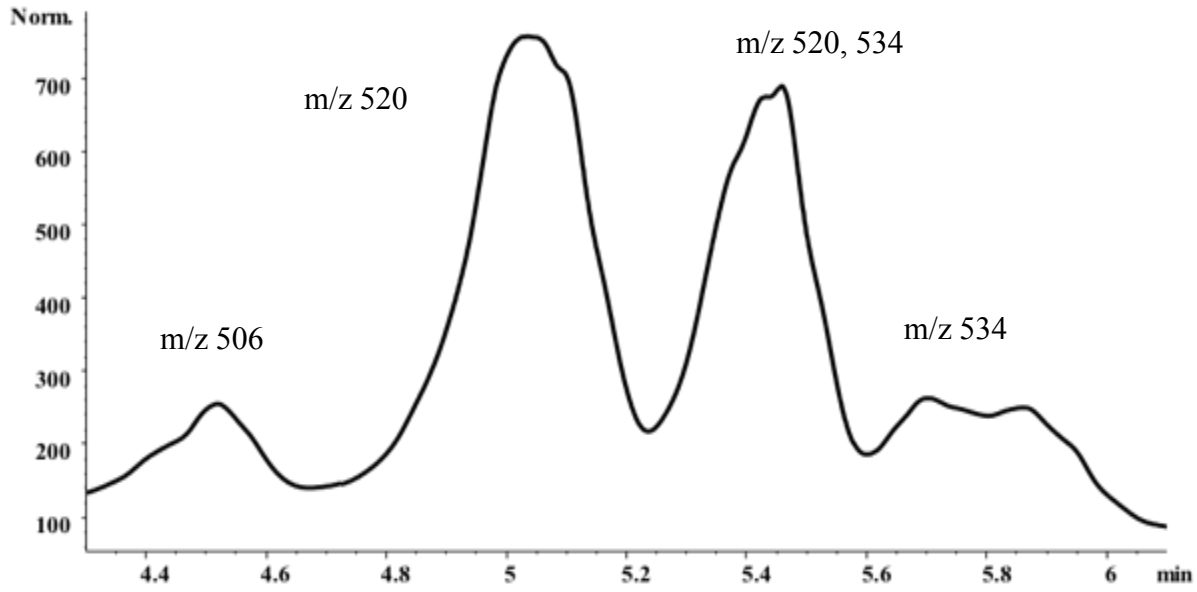


Figure 4-15 Semi-preparative HPLC chromatogram (4.3-6.1 min) of the *C. sativa* extract.

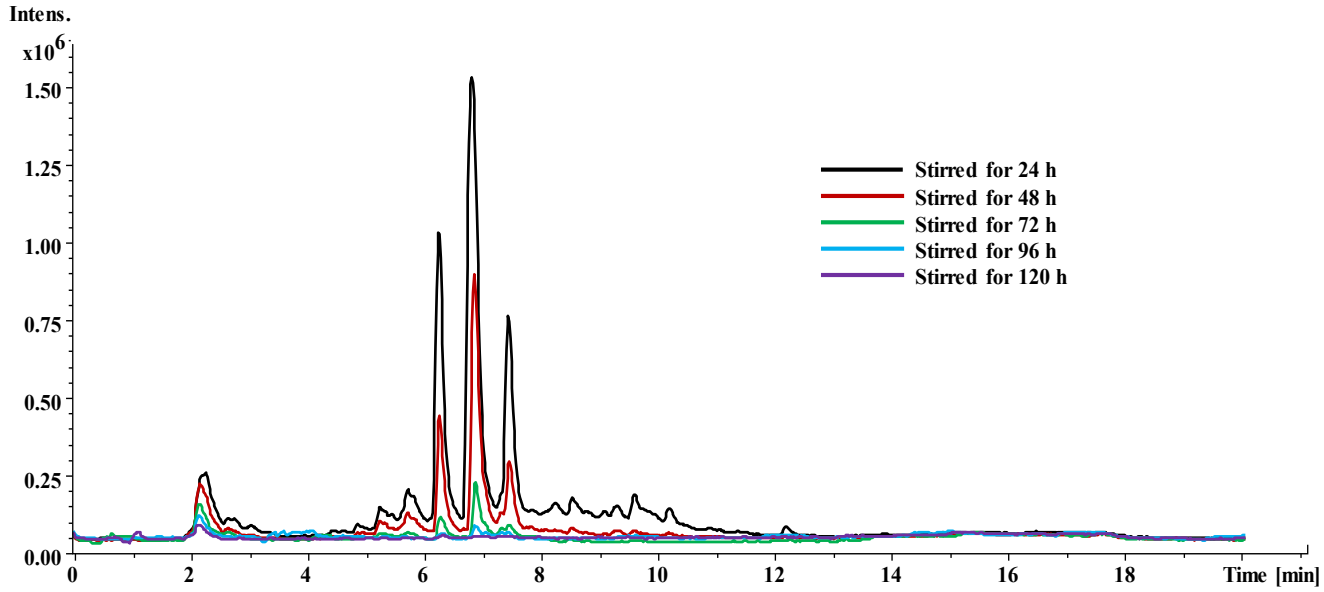


Figure 4-16 Overlaid total ion chromatograms of *C. sativa* oil solid extracted for 24, 48, 72, 96, and 120 h.

4.5.2 Glucosinolates isolation using preparative HPLC

A preliminary experiment was conducted, in order to investigate the suitable volume of solvent and extraction times for glucosinolates from the processed oil solids. In this experiment, the material (0.5 g) was mixed with 80% (v/v) aq. ethanol (10 mL) and stirred at room temperature for 24 h. This mixture was centrifuged and the supernatant concentrated and re-constituted with HPLC starting gradient solution. The remaining pellet from centrifugation was mixed with 80% (v/v) aq. ethanol (10 mL) and stirred at room temperature for another 24 h, followed by the same techniques of centrifugation, concentration and re-constitution. This procedure was repeated until the same oil solids had been extracted for a total of 120 h with 80% (v/v) aq. ethanol of a total amount of 50 mL. The re-constituted samples, representing an extraction time of 24, 48, 72, 96, and 120 h, were subject to HPLC-ESI-MS analysis. The mass spectral results from this experiment, which was conducted in duplicate, were consistent. The overlaid total ion chromatograms of the re-constituted extracts (Figure 4-16**)** show that *C. sativa* glucosinolates could still be detected after the material had been extracted for 96 h, but hardly detectable after 120 h.

During the 43 min method when the isolation was performed on preparative HPLC, the retention of components in the complex matrix might have changed. Each concentrated ethanolic extract was dissolved in 4 ml of the starting gradient of mobile phases and loaded into a 1.9-ml injection loop. At the beginning, twenty-eight to thirty fractions were collected every minute after each injection, following a void volume of approximately 130 ml. It has been noted that *C. sativa* glucosinolates have very weak UV absorbance, and the isolation using preparative HPLC was dependent on the observation of their co-eluting compounds on the monitor, which have similar polarity to them. A portion (5 μL) of each fraction collected was analyzed by LC-MS before fraction enrichment. Eventually fractions that were collected at retention times 25.5 to 26.5 min, 27.5 to 28.5 min, and 31.5 to 32.5 min were shown to contain *C. sativa* glucosinolates with m/z 506, 520, and 534, respectively (Figure 4-17), from which components with m/z 294, 741, 593,463, and 623 were also detected.

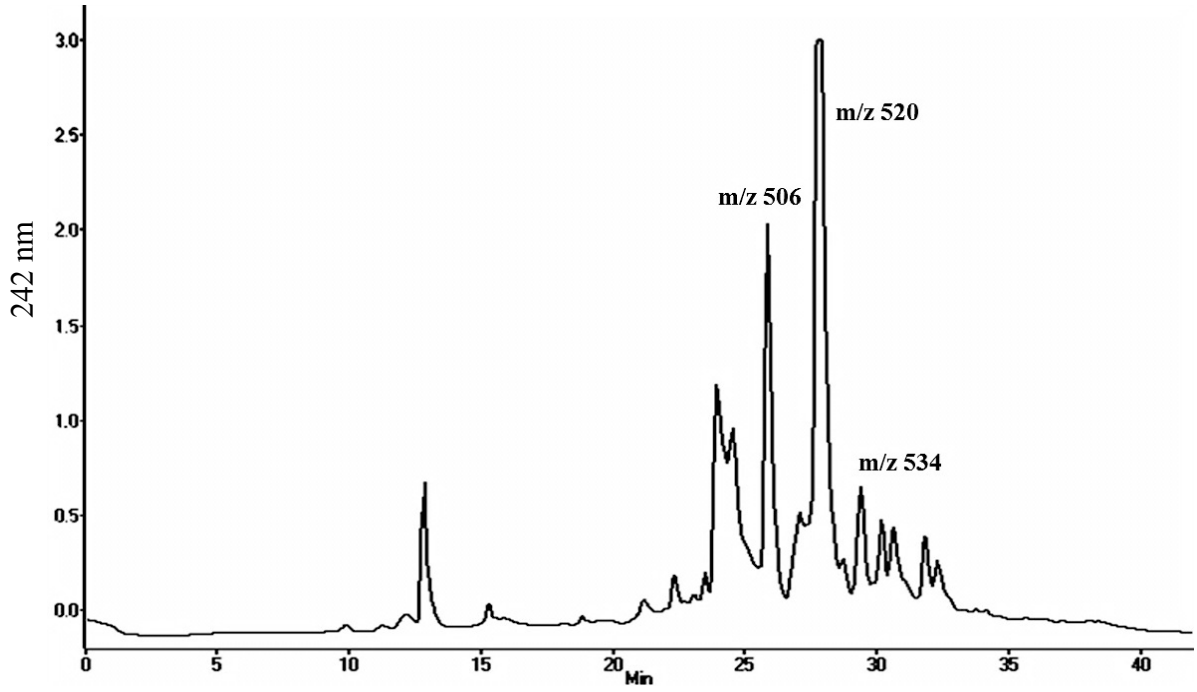


Figure 4-17 Preparative HPLC chromatogram of *C. sativa* extract.

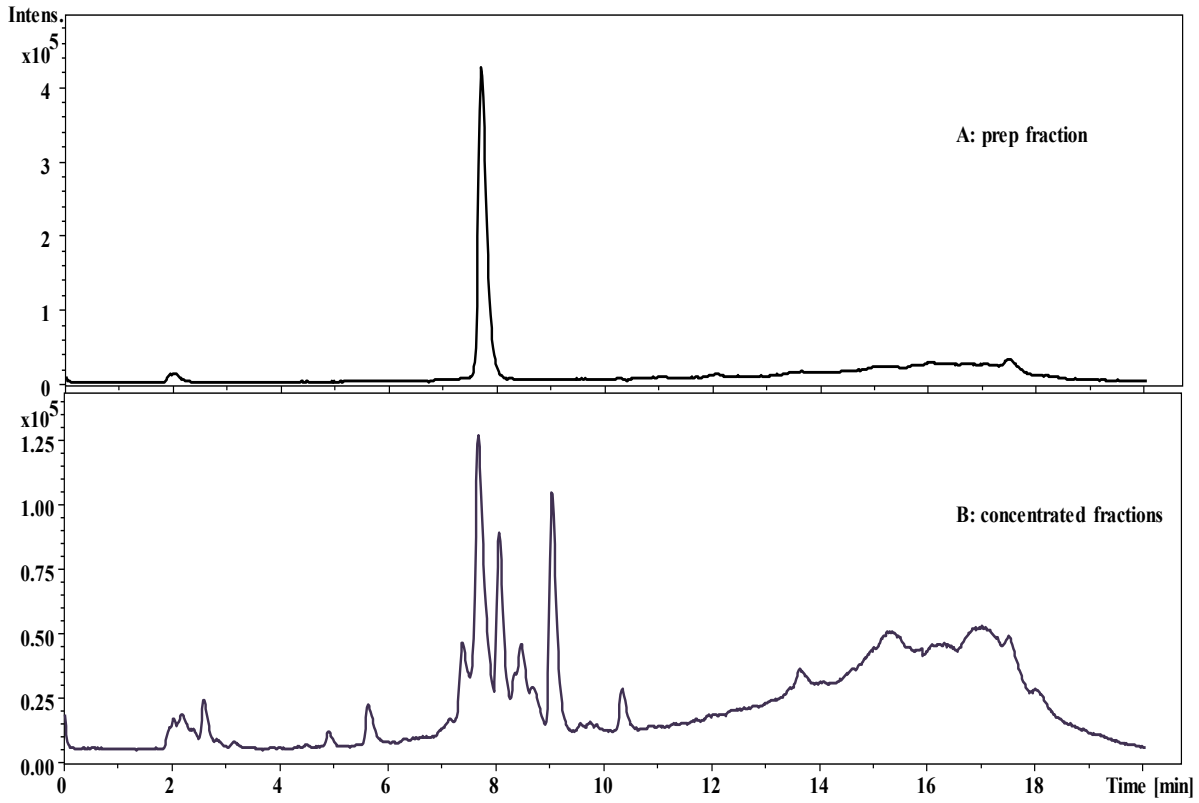


Figure 4-18 Total ion chromatograms of glucosinolate with m/z 534. (a) One of the fractions collected from preparative HPLC; and (b) an enriched sample concentrated from multiple fractions using airflow.

During enrichment of each fraction, it was observed that these compounds tended to degrade, and the degree of degradation was enhanced by fraction enrichment through the airflow (Figure 4-18). Concentration using rotary evaporator connected to a vacuum was performed instead. It was anticipated that constituents in the mobile phase, which were water, formic acid, and acetonitrile, could be easily removed with the assistance of vacuum pressure, and deterioration from oxygen could be avoided. Besides concentration directly using vacuum, the idea of neutralizing the acidic glucosinolates with a counter-ion was also practiced and worked well. NH_4OH (1 mg/mL in MeOH) or $NaHCO_3$ (1 mg/mL in MeOH) were thoroughly mixed with fractions from preparative LC at a ratio of 1:1 (v/v) before concentration under vacuum pressure.

With the increasing interest in the chemoprotective attributes of glucosinolates and their breakdown products, quantities of purified standards are required for biological and clinical studies. While these three *C. sativa* glucosinolates are not yet commercially available at the time of study, the isolation and purification technique described above provided a potential direction for their preparation and purification, which would allow future studies of their hydrolysis-derived isothiocyanates. For future experiments, it is recommended that preparative HPLC be conducted prior to semi-preparative HPLC, which would enable further purification of the semi-purified fractions.

4.6 NMR analysis of camelina glucosinolates

The HPLC-ESI-MS method described was able to identify *C. sativa* glucosinolates in complex plant extracts, and the analysis result also suggested the relatively weak UV absorbance of these compounds, which made further quantitation from a UV detector unlikely. An HPLC refractive index (RI) detector was not found to be useful for the analysis of *C. sativa* glucosinolates either. NMR spectrometry was an alternative, which has been embraced for its accuracy in positively characterizing the structures of organic compounds in biological samples, as well as the ability to provide a possible solution for performing quantitative analysis when an appropriate internal standard is applied.

4.6.1 NMR confirmation of glucosinolates in camelina fractions

While authentic standards of the three *C. sativa* glucosinolates were not yet available at the time of experiment, standards of their structurally similar analogue glucoraphanin could be

obtained. NMR characterization of glucoraphanin standard would be able to provide reference information for confirmation of *C. sativa* glucosinolates from plant extract. The ^{1}H NMR spectrum (500 MHz) of glucoraphanin standard is presented in Figure D-1 (Appendix D), from which general consistency with few variations were observed compared to previous analysis of the compound that was isolated elsewhere (Prestera *et al*., 1996). Protons attached to the side chain are separated from those attached to the thioglucose group, which enables further molecules confirmation. Signal overlapping occurred to some glucose protons, such as those triplets from H2-G and H4-G. The strongest signal at 2.46 ppm came from the resonances of methylsulfinyl group (SO-CH_3) at the end of the side chain. In the report by Prestera *et al*. (1996), the same group of protons appeared at chemical shift at approximately 2.71 ppm, which also illustrated how the SO chirality would result in unequal chemical shifts of the methylene group (CH_2) attached to it. Due to the relatively high purity of glucoraphanin standard used and the same experimental condition followed in current work, it is believed that the NMR information thereafter obtained is more convincing and relevant, and could be used as the reference for the following *C. sativa* glucosinolate structural conformation.

Semi-purified fractions from the semi-preparative HPLC work were used as the enriched samples for determination of the *C. sativa* glucosinolates. As can be seen from the spectrum (Figure D-2, Appendix D), the protons from the thioglucose group are well separated from the aliphatic side chain protons, which show similar chemical shifts with that of the glucoraphanin standard. Other resonances recorded also revealed clear correlations to the side chain of the glucosinolate (GS9) despite some minor impurities observed in the fraction. Resonance at chemical shift 2.56 ppm had the strongest signal, which could be traced back to the three protons in the methylsulfinyl group (SO-CH_3), given that the similar methyl group attached to SO of glucoraphanin has resonance at 2.46 ppm. If this assumption would be confirmed positively, not only the resonance at 2.56 ppm could be used for identification or further quantitation of the compounds in various plant fractions, it would also suggest that *C. sativa* glucosinolates have very similar SO functions with glucoraphanin.

In a more complex sample, the resonance at 3 to 4 ppm would have been occupied by other components in the glucosinolate-containing extract, which had occurred before (Belliveau and Romero-Zerón, 2010). While the peak detected at 2.56 ppm on ^{1}H NMR could be referenced to the methylsulfinyl group (SO-CH_3) in an enriched sample, in a more complex sample it was

necessary to clarify between this group and other non-substituted S-methylene groups ($S\text{-}CH_2$) from other components. The *C. sativa* oil solid extract, representing a less glucosinolate enriched material, was re-constituted in D_2O and subject to ^{1}H NMR, ^{13}C NMR ($C_{13}CPD_{90}$) combined with two dimensional heteronuclear multiple-quantum correlation (HMQC), distortionless enhancement by polarization transfer (DEPT) as well as attached proton test (APT) analyses. In the HMQC experiment, the protons recognizable at 2.56 ppm could be coupled with the carbon at 36.3 ppm, the latter one of which falls into the region (20-50 ppm) where normal substituted methylene groups could take place. The negative peaks from APT analysis confirmed the peak at 2.56 ppm represented methyl and not methylene protons (Figure D-3, Appendix D).

4.6.2 Quantitative NMR analysis for glucosinolates

As demonstrated in literature review, one of the advantages of quantitative NMR is that it omits the necessity of using authentic standards for calibration when an appropriate internal standard is found. This is highly useful for current work when *C. sativa* glucosinolates are not yet available. Glucosinolates quantitation using NMR method is not as common as that of the chromatography methods, but there is evidence that the technique established great potential for the purpose (Pauli *et al*., 2012). A previous study illustrated the identification of sinigrin by ^{1}H NMR, from which the change in the compound's level during enzymatic reactions could also be observed; authors of this study reported that sinigrin could be effectively observed at 6.04 and 5.30 ppm, using D_2O as the reference peak at 4.79 ppm (Belliveau and Romero-Zerón, 2010).

As a preliminary experiment, sinigrin standards in the range of 1 - 16 mg/mL were prepared in DMF solution (1 mg/mL in D_2O) and analyzed on ^{1}H NMR to validate that the technique is suitable for quantitation of glucosinolate. Mohn *et al.* (2007) illustrated that the compound could be measured at 4.5 ppm for its anomeric hydrogen using 1,3,5-trimethoxybenzene as an internal standard. In this experiment, DMF as an internal standard was tested for the linear calibration of glucosinolates and chemical shifts at 2.60 ppm, 2.75 ppm, and 7.70 ppm were found representative as reference signals for measuring sinigrin (Figure D-4, Appendix D). At 7.70 ppm the highest accuracy was achieved (93.77% to 100.61%) in measuring sinigrin at 3.45 ppm. Other NMR-active nucleus of sinigrin could also be measured at 3.65 ppm, 4.80 ppm, 5.05 ppm and 5.80 ppm. The NMR spectrometry was therefore proved to be sufficiently sensitive and accurate to pursue the quantitation of the following work.

NMR analysis of the *C. sativa* oil solid suggested that the relative amount of

glucosinolates in *C. sativa* seed material may be comparatively low, which was also agreed with previous reported data regarding glucosinolate level in the seed (Schuster and Friedt, 1998; Matthäusa and Zubr, 2000). This led to the decision of using DMF at concentration 0.05 mg/mL. Quantitation of glucosinolates could be achieved by a combination of proton NMR analysis and LC-MS analysis, which determined the amount of total and individual glucosinolates, respectively.

4.7 The fate of glucosinolates during primary processing of *Camelina sativa* seed

4.7.1 Oil yield and oil content of camelina seed

C. sativa seed was pressed in triplicate in order to calculate the oil yield. Preliminary experiment showed that while flow rate was adjustable, too high a flow rate caused oil to accumulate inside the expeller. Crude oil 308.8 ± (8.9) g and seed meal 669.6 (± 6.4) g were obtained after pressing of 1000.0 g of *C. sativa* seed. The weight loss observed may be due to materials remaining in the expeller after pressing. The calculated crude oil yield was therefore 30.88 % (± 0.89). Oil solids were recovered from crude oil for later analysis of glucosinolates as described in section 3.5.2. A total of 2.7 g of dry oil foot was produced from the crude oil pressed from 3.0 kg of *C. sativa* seed.

Oil content of *C. sativa* whole seed and seed meal using Goldfisch extraction was 35.13% (± 0.30) and 15.30% (± 0.16), respectively. The oil content obtained from this study was measured based on an "as is" basis. Previously, published data regarding the *C. sativa* seed oil content was determined on a dry basis, such as a recent study by Gugel and Falk (2006), which reported 38 to 43% of oil content, and an earlier one by Budin *et al*. (1995), which reported 29.9 to 38.3% of oil content.

4.7.2 Dehulling of camelina seed

The purpose of dehulling, as discussed in the literature review, is to remove the hull and maintain as much cotyledon as possible so as to improve the oil yield and the quality of both oil and seed meal. A multi-step method for processing the rapeseed was described by Rass and Schein (2010), in which the pressed seed cake was subject to grinding for more than one time before distribution. In this patented method, dehulling was realized by first sorting rapeseed based on seed size then disrupted in a rolling milling; the disrupted seed was further sorted by screening and sifting to obtain a low fiber material before further processing (Rass and Schein,

2010). It is plausible that this method can be applied to the processing of *Brassica carinata* seed after suitable adjustment, given that seed from this species has similar size and appearance to rapeseed. On the other hand, *Camelina sativa* seed had a very different anatomy: the seed size is much smaller (Small, 2013) than that of rapeseed varieties, and the adhesion between the seed hull and cotyledon is considered to be much tighter, which suggests that the translation from the patent method for rapeseed processing to *C. sativa* seed unlikely. The aim of the current work was to experiment on the dehulling processing of *C. sativa* seed, and to further design a pneumatic system for the efficient dehulling of this oilseed based on the experimental data.

4.7.2.1 Dehulling of camelina seed in laboratory scale

There were a variety of settings in the disc mill that represent the space between the two grinding discs for adjusting the size and fineness of the ground material. The larger the number in the setting, the more space there is between the two discs and therefore a coarser material will be produced. After preliminary adjustment, settings were narrowed down in the range of 4.3 to 6.3 (with the disc on the outside fixed to the end), from which the ground seed was considered suitable for dehulling through air separation. As representatives, ground seed treated from the setting 4.3 and 6.3 were sieved through an 850-µm test sieve (No. 20, VWR, West Chester, PA, USA), then through a 425-µm test sieve (No. 40, VWR, West Chester, PA, USA), respectively. Materials remaining on each sieve were collected and subject to dehulling. In addition, materials that stayed on the 850-µm test sieve from setting 6.3 were ground a second time at setting 4.3 on the disc mill for air aspiration.

During the dehulling operation, a portion of *C. sativa* ground seed (~10 g) treated from the disc mill and sieving was tested for each run. Hull was collected efficiently during the first few trials; however, the finer cotyledon would mix with the hull with stronger treatment. To obtain the largest possible amount of underlying tissues from the dehulling process, aspiration was halted once the light color seed meat was observed mixing with the hull on the collector. A considerable amount of hull was still mixed with the cotyledon after the dehulling treatment from the seed cleaner (Figure 4-19). To assess the milling efficiency, these mixtures were collected, and their oil content (Goldfisch), moisture content and crude protein content were measured (Table 4-6).

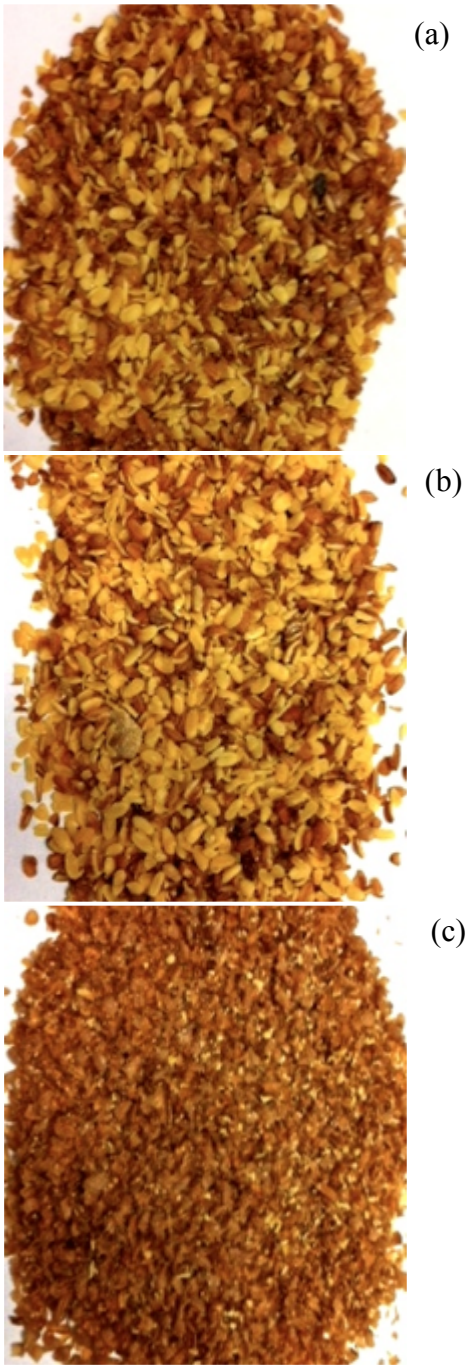


Figure 4-19 *C. sativa* seed from a dehulling treatment. (a) Seed ground from disc mill (treatment 5 as described in footnote of Table 4-10); (b) cotyledon part of the ground seed after dehulling; and (c) seed hull collected after dehulling.

Table 4-6 Properties of *C. sativa* seed materials before and after dehulling treatment

Before dehulling					
Treatment	(n=3)	Oil content (%)	Moisture content (%)	Crude protein (%)	Hull (%)
1[a]	Mean	28.13	4.67	28.71	14.45
	SD	2.06	0.24	0.60	0.06
2[b]	Mean	28.70	4.80	29.41	13.23
	SD	0.06	0.08	0.44	1.08
3[c]	Mean	28.95	4.67	28.63	10.44
	SD	0.50	0.07	0.40	0.36
4[d]	Mean	30.81	4.64	28.62	13.22
	SD	0.35	0.04	0.30	0.44
5[e]	Mean	29.84	4.61	29.67	12.27
	SD	0.57	0.09	0.34	0.22
	Overall	29.29	4.68	29.00	12.44
	SD	1.29	0.13	0.59	1.48
After dehulling treatment					
Treatment	(n=3)	Oil content (%)	Moisture content (%)	Crude protein (%)	Hull crude protein (%)
1[a]	Mean	35.38	4.09	31.04	20.58
	SD	0.70	0.06	0.36	0.29
2[b]	Mean	30.83	4.15	30.20	23.38
	SD	0.74	0.02	0.63	0.34
3[c]	Mean	30.71	4.18	29.79	25.26
	SD	0.30	0.05	0.69	0.17
4[d]	Mean	35.55	3.79	31.12	19.95
	SD	0.25	0.13	0.75	0.42
5[e]	Mean	32.41	4.21	31.24	21.72
	SD	0.16	0.04	0.50	0.49
	Mean (n=15)	32.98	4.08	30.68	22.18
	SD	2.23	0.17	0.78	2.02

[a] The sample from treatment 1 refers to *C. sativa* seed ground from setting 4.3 on the disc mill as described, passed through an 850-µm test sieve, and stayed on a 425-µm test sieve.

[b] The sample from treatment 2 refers to *C. sativa* seed ground from setting 4.3 on the disc mill as described, and stayed on an 850-µm test sieve.

[c] The sample from treatment 3 refers to *C. sativa* seed ground from setting 6.3 on the disc mill as described, and stayed on an 850-µm test sieve.

[d] The sample from treatment 4 refers to *C. sativa* seed ground from setting 6.3 on the disc mill as described, passed through an 850-µm test sieve, and stayed on a 425-µm test sieve.

[e] The sample from treatment 5 refers to the sample from treatment 3 that were ground again from setting 4.3 on the disc mill.

The average percentage of hull separated from *C. sativa* ground seed from five different grinding treatments was 12.44% (± 1.48). This is in agreement to the result obtained from CETIOM, which reported the hull percentage in rapeseed to be 12-13% (Mulder *et al*., 2012). Percentages of hull obtained were higher when the seed was first ground at setting 4.3 prior to air separation. The seed sample from treatment 1 resulted in the highest amount of hull separated, which was 14.45% (± 0.06). Hull obtained were comparatively less when the seed was ground at setting 6.3 prior to air separation. The seed sample from treatment 3 had the lowest amount of hull separated, which was 10.44% (± 0.36), but regrinding samples in the disc mill helped to improve the dehulling efficiency (treatment 5). This experiment demonstrated that the grinding process was critical in reducing the adhesion between seed hull and cotyledon, and that using a setting of 4.3 in the disc mill as described helped to achieve a *C. sativa* ground material that made the later air separation of hull easier.

Since seed hull is normally considered to contain mostly fiber and only a small amount of oil and crude protein, they are thought to be of less importance when the target product is oil and protein meal; therefore, oil content is an essential parameter in assessing dehulling efficiency. The oil content of *Camelina sativa* whole seed (ground) was 35.13% (± 0.30), as indicated in section 4.7.1. The grinding treatment in the disc mill resulted in a decrease of the oil content tested to 29.29% (± 1.29). The major reason may be that the ground materials from disc mill were wrapped and extracted for oil directly, while the whole seed was ground in a coffee mill to finer powders, which assisted in a more thorough oil extraction. All *C. sativa* fractions of the dehulling experiments had not been ground a second time; the results of the oil extraction were considered comparable. After air separation, the average oil content increased to 32.98% (± 2.23). The seed sample from treatment 1 had the highest increase (20.76%), resulting in oil content of 35.38% (± 0.70), which was also the second highest oil content obtained after dehulling. The seed sample from treatment 4 had the highest oil content after dehulling, which was 35.55% (± 0.25), with the second highest increase (15.35%) compared to the material before dehulling from air separation. These two samples were both materials that passed through the 850-µm test sieve after grinding, suggesting once again that a finer material within a range helped to improve the dehulling process, as well as a more complete oil extraction. The lowest increase in oil content (6.06%) after air separation occurred in sample from treatment 3; this was consistent with the low hull obtained from the sample, indicating that *C. sativa* seed materials ground in this setting

were too coarse for the seed structures to separate. A regrinding process of the sample in the disc mill helped to increase its oil content after air separation, which was also indicated by an increase amount of hull separated (treatment 5). Therefore, the oil content of the seed fractions could be used as an indicator of dehulling efficiency.

In terms of crude protein content, the *C. sativa* whole seed (ground) tested contained 28.76% (± 0.17). Grinding the seed in the disc mill resulted in a slight increase of the value to 29.00% (± 0.59). After air separation, the average crude protein content increased to 30.68% (± 0.78). The dehulling treatment by aspiration increased the crude protein content significantly (p <0.05). Sample from treatment 5 had the highest crude protein content after dehulling, which was 31.24% (± 0.50) while sample from treatment 3 had the lowest crude protein content among the six samples tested, which was 29.79% (± 0.69). The highest increase of crude protein content after air separation was 8.75%, which was observed in the seed sample from treatment 4, reaching 31.12% (± 0.75) crude protein content. It was also noted that a considerable amount of crude protein was measured in the seed hull; an average of 22.18% (± 2.02) was calculated, which explained the inconsistency between the amount of hull obtained from each sample and the increase in protein content after dehulling. Despite the fact that a number of seed meat was also mixed with the hull fractions, the high value of protein content measured suggest that the hull itself contributed to a considerable portion of protein.

In terms of moisture content, the *C. sativa* whole seed (ground) tested contained 5.16% (± 0.06). The moisture content of the material after grinding in the disc mill dropped to an average of 4.68% (± 0.13). After air separation, the average moisture content dropped to 4.08% (± 0.17). The decrease in moisture content could be attributed to evaporation of water from the broken seed. The highest reduction of moisture content reached 18.44% from sample from treatment 4, with moisture content 3.79% (± 0.13). The seed sample from treatment 5 had the highest moisture content among all five samples after dehulling, which was 4.21% (± 0.04).

The dehulling experiments on *C. sativa* seed was conducted in a small scale in this study, which confirmed the plausibility of such treatments. Pre-grinding in the disc mill was shown necessary to produce a loose mixture of seed hull and cotyledon. A comparatively finer ground material, which was achieved by a smaller setting in the disc mill used, and proper sieving, made the separation of the hull from the mixture easier, which was indicated by an apparent increase in oil content after dehulling. On the other hand, material that was ground too finely were not be

suitable for dehulling using air separation.

4.7.2.2 Designing of a pneumatic system for camelina dehulling

Currently there is no processing system available that is designed specifically for the dehulling of *Camelina sativa*. Knowledge on the physical properties of a given agricultural material, such as the mean diameters, thousand seed mass, bulk density, terminal velocity and projected area is essential for efficient design of equipment for handling, transportation, sorting, separation, and processing of the agricultural material. Research works have been reported on the physical properties of the seed of wheat, barley, sunflower, lentil (Güner, 2007), soybeans (Innocentini, 2009), and rapeseed (Çalışır *et al*., 2005; Izli *et al*., 2009). Little has been reported on the physical properties of *Camelina sativa* seed.

Terminal velocity is one of the most important parameter in designing a pneumatic system for dehulling oilseed. Ideally, a system that is able to control the air velocity to be lower than that of the cotyledon of the *C. sativa* seed and higher than that of the seed hull and other unwanted fines can achieve a total dehulling. As was observed from the dehulling experiments described in section 4.7.2.1, some lighter seed meat would be blown up to the cyclone with the hull after a few trials while certain amount of hull with higher weight remained at the bottom of the cylindrical column, which led to incomplete dehulling. The reason for this was the overlapping of the terminal velocity of the seed meat and hull in the ground materials, which could be traced back to the one-time grinding processing the disc mill that resulted in similar size of different parts of the *C. sativa* seed. A subsequent sieving step apparently improved the dehulling efficiency (treatment 1 and 4), and so did a re-grinding step (treatment 5), which inspired that a pneumatic system designed to involve multi sieving and grinding steps that would be added based on the size distribution of the seed materials, would be likely to help get closer to the complete dehulling of *C. sativa* seed. Terminal velocity of a spherical particle can be predicted according to Equation 4.3 (Tilton, 1997). The actual terminal velocity of oilseed can also be measured through a "wind column", as was described by Güner (2007).

$$v_t = \sqrt{\frac{4 \times d_p \times (\rho_p - \rho) \times g}{3 \times C_D \times \rho}} \tag{4.3}$$

Where v_t refers to the terminal velocity, d_p refers to the particle diameter, ρ_p refers to the particle density, ρ refers to the density of surrounding fluid, g refers to the acceleration of

gravity, and C_D refers to the drag coefficient of the rigid spherical.

Generally, the density of seed hull is higher than that of the seed meat, since the previous one mainly consists of fiber while the latter one contains considerable amount of oil. The flatter shape of seed hull allows for the larger projected area that eventually lowers the drag force and the terminal velocity. In practice, the terminal velocity of the biological material in a system is affected by many factors, including the configuration of the sample, such as the shape, size and moisture content, other physical-chemical properties such as the density and viscosity, and the concentration of the samples inside the system (Innocentini, 2009). This was in agreement to the experimental observation from the current work. While the setting of 4.3 to 6.3 was determined for crushing the *C. sativa* whole seed before dehulling, a closer look at the sieving data obtained from different setting on the disc mill helped to reveal the particle size distribution of the *C. sativa* ground seed. A portion of whole seed (~5 g) was fed into the conical hopper for grinding. The ground material treated from each setting were collected and passed through an 850-μm test sieve (No. 20. VWR, West Chester, PA, USA), followed by a 425-μm test sieve (No. 40. VWR, West Chester, PA, USA) and finally a pan. Materials remaining on each sieve were collected, weighed, and the mass ratio (%) was calculated (Table 4-7).

As can be seen from the graphical demonstration (Figure 4-20, a), although the disc mill setting was narrowed down to a small range, the distribution observed was relatively diverse, suggesting that even a minor deviation during milling would result in great difference in the size of different composition in the mixture. Selected treatments were introduced to the seed cleaner for dehulling and it was shown that for one-time grinding, smaller size of the material that achieved from more intense treatment in the disc mill benefited higher dehulling efficiency (treatment 1 and 4 in Figure 4-20, b). In addition, re-grinding of the material improved the dehulling (treatment 5, Figure 4-20, b).

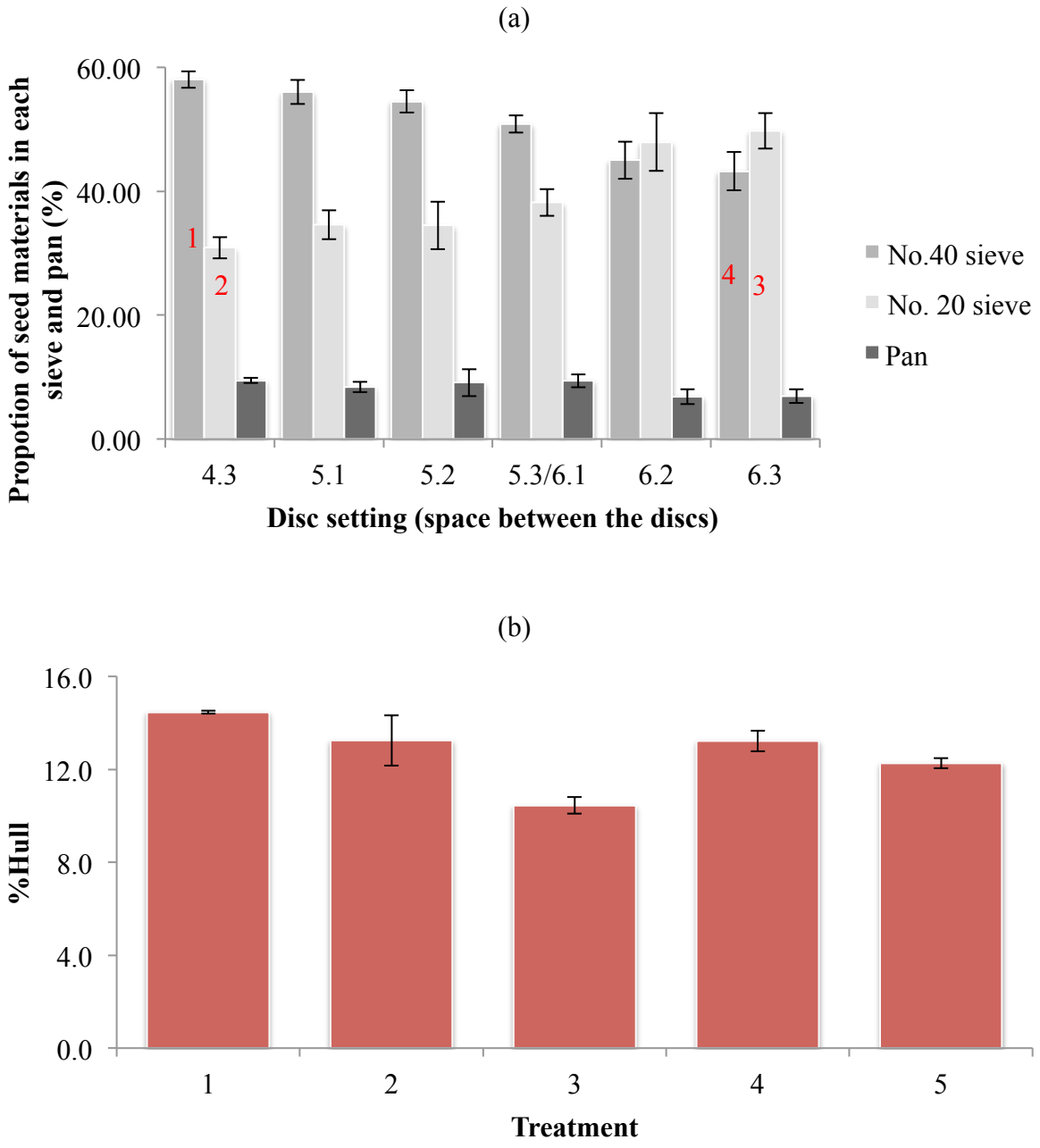


Figure 4-20 Particle size distribution and dehulling of *C. sativa* seed. (a) Mass proportion of ground seed in different sieves and pan; and (b) percentage of hull obtained from selected treatments.

Table 4-7 Grinding efficiency of different settings on the plate mill

Setting	(n = 4)	Mass ratio[a] (%)		
		850-µm test sieve	450-µm test sieve	Pan
6.3	Mean	49.78	43.23	6.93
	SD	2.88	3.08	1.10
6.2	Mean	47.94	45.03	6.85
	SD	4.66	3.01	1.19
6.1/5.3	Mean	38.21	50.85	9.41
	SD	2.17	1.39	1.05
5.2	Mean	34.49	54.49	9.11
	SD	3.85	1.78	2.20
5.1	Mean	34.59	55.99	8.41
	SD	2.33	1.94	0.87
4.3	Mean	30.90	58.03	9.45
	SD	1.74	1.33	0.41

[a] Mass ratio was calculated by dividing the weight of materials stayed on the sieve/pan by the weight of the material before sieving

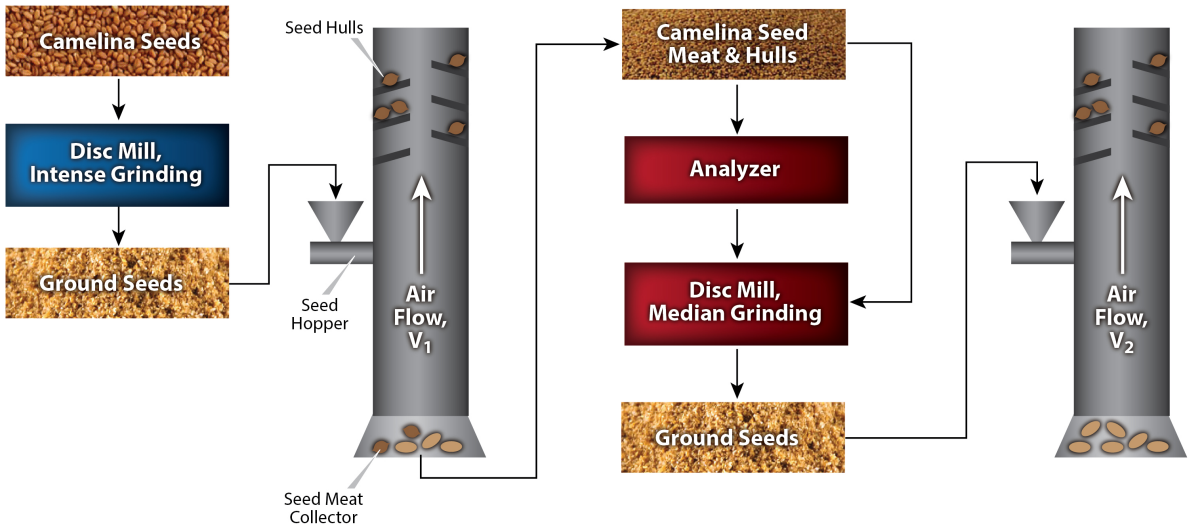


Figure 4-21 Schematic of a proposed dehulling system for *C. sativa* seed.

Figure 4-21 is a simplified graphical illustration of a proposed pneumatic system for dehulling of *C. sativa* seed. Intact *C. sativa* seed is first ground in a comparatively intense mode in the disc mill, which has been shown to assist in the dehulling efficiency. The collected ground materials that remain at the bottom of the seed meat collector after the first separation are subject to a range of analysis that aims to evaluate the proportion of the hull in the mixture. Here the measurement using specific chemical marker is proposed. It was shown in a previous study on canola oil, that the composition was different between the hexane insoluble fraction of the seed hull lipid and the oil, with the variance mainly in the amount of shorter-chain alcohols and waxes (Liu *et al*., 1996). Similar phenomenon may exist in the *C. sativa* hull lipid that requires further examination. Alternative treatment might be added to the system: while it has been reported that moisture content is influential on the terminal velocity, it is likely that the change in moisture content tends to change the true density of the material. Since porosity is a function of the true density and bulk density of an object, a chilling treatment as the pre-conditioning might be able to alter porosity of the oilseed, thus increase the terminal velocity of the oil-rich seed cotyledon. In addition, while the system encourages multiple grinding of the seed material that will be followed by dehulling using adjustable air velocity, it has been shown that oilseed quality would be compromised in a pneumatic conveying system, and the damage would be influenced by the increase of air velocity in the system (Güner, 2007). Here subsequent measurement is suggested to monitor the quality of *C. sativa* seed after multiple dehulling treatments, in which a vigour test can be conducted on the seed and served as a measure of the quality of the materials (ISTA, 1985).

4.7.3 Glucosinolate distribution in *Camelina sativa* seed fractions

4.7.3.1 Quantitation of camelina glucosinolates using one internal standard

C. sativa whole seed tested in this project had a moisture content of 5.16 (± 0.06) % as presented in section 4.7.2. To determine the appropriate times of extraction, ground seed (50 mg) were extracted with 80% (v/v) aq. ethanol (1.5 mL) for a total of five times. The signals of glucosinolates protons were barely observable after the third time of extraction. Thereafter three extractions with 1.5 mL were used for recovering *C. sativa* glucosinolates. The distribution of *C. sativa* glucosinolates during the processing of the oilseed is shown in Table 4-8.

Table 4-8 Glucosinolate distribution during primary processing of *Camelina sativa*

Material	(n=3)	Glucosinolate distribution (μg/mg)			
		GS9	GS10	G11	Total
Whole seed	Mean	3.06	9.03	2.04	14.12
	SD	0.15	0.41	0.06	0.61
Seed meal	Mean	5.52	15.20	3.55	24.27
	SD	0.28	0.75	0.18	1.21
Defatted seed	Mean	6.64	17.97	4.35	28.96
	SD	0.35	0.91	0.17	1.42
Defatted meal	Mean	7.51	19.51	4.79	31.81
	SD	0.17	0.57	0.10	0.80
Oil foot	Mean	7.65	19.79	4.78	32.21
	SD	0.49	1.09	0.24	1.82
Crude oil extract	Mean	6.22	15.32	3.28	24.81
	SD	0.76	0.39	0.56	1.71
Crude oil[a]	Mean	1.90	4.65	1.00	7.56
	SD	0.49	0.75	0.31	1.54
Seed hull	Mean	1.68	6.43	1.78	9.88
	SD	0.12	0.38	0.10	0.59

[a] One outlier removed; in unit of μg/g.

Total glucosinolate level in *C. sativa* whole seed was 14.12 (± 0.61) μg/mg on an "as is" basis, which could also be expressed as 27.17 μmol/g. Glucocamelinin (GS10) is the major glucosinolate among all seed materials tested, taking up to 65% of the total glucosinolate content. The content of GS9 is consistently higher than that of GS11 in all samples tested except in seed hull. The results obtained are in accordance with those presented by Schuster and Friedt (1998), who reported the content of total glucosinolates in different genotypes of *C. sativa* seed to fall into the range of 13.2 to 36.2 μmol/g dry seed, within which glucocamelinin occupied about 65% of them. But in another published study, the content of GS11 measured was higher than that of GS9 (Matthäus and Zubr, 2000). In the published study, total glucosinolates in seed determined was 9 to 19 μmol/g, and the corresponding seed meal contained 14.5 to 23.4 μmol/g total glucosinolates (Matthäus and Zubr, 2000). The total glucosinolate content in seed meal obtained in the current study was much higher than that, with 24.27 (± 1.21) μg/mg, which could also be expressed as 46.69 μmol/g. The published study mentioned above used the technique of desulfoglucosinolates, in which glucosinolates in the crude extract was treated with sulfatase to remove the sulfate group prior to analysis by HPLC. In the current study, the desulfation step was omitted and a more simple way of sample preparation was carried out. The combined

analysis of proton NMR and HPLC-ESI-MS not only accelerated the measurement, but also improved the accuracy. It was also noted that the glucosinolate content in both *C. sativa* whole seed and seed meal was approximately 50% less than the sinigrin level measured in *B. carinata* seed.

Oil foot contained the highest level of glucosinolates (32.21 ± 1.82 μg/mg) among the seed materials tested, which proved again this fraction of the seed was a good candidate for isolation of the compounds. The glucosinolate level in *C. sativa* crude oil was calculated from the yield of crude oil extract, with the extraction step described in section 3.3.3**.** Eventually 152.50 mg of extract concentrate was produced from 502.65 g of crude oil (one outlier removed), resulting in a yield of 0.03%. This result was lower than the crude oil extract yield obtained from *Brassica carinata* seed, by approximately 20%. Estimated sinigrin content in defatted *B. carinata* seed in section 4.3.3.2 was calculated in two ways: with and without inclusion of the sinigrin content in crude oil. Using the same methods, estimated total glucosinolate content in defatted *C. sativa* seed would be 17.67 (± 0.05) μg/mg, or 21.77 (± 0.10) μg/mg when the content in crude oil fraction was omitted given the oil content of *C. sativa* seed determined in section 4.7.1 being 35.13% (± 0.30). In this experiment, the defatted seed from Goldfisch extraction were extracted for glucosinolates, and the measured value was 28.96 (± 1.42) μg/mg, which was higher than both the estimated ones, which could be explained by the small size of the defatted material that increased the surface area for extraction. The same happened to *C. sativa* seed meal, whose oil content was determined to be 15.30% (± 0.16) that resulted in lower estimated total glucosinolate contents in defatted seed meal (27.29 ± 0.054 μg/mg or 28.65 ± 0.05 μg/mg) than the experimental result, which was 31.81 (± 0.80) μg/mg; the measured glucosinolate content in *C. sativa* defatted meal was more than twice as high as those reported previous (Berhow *et al*., 2013). During measuring the sinigrin distribution in the processing of *B. carinata* seed in section 4.3.3.2, it was concluded from experimental results and extrapolation that the crude oil extract, oil solids and seed meal could be categorized into one. This part of the experiment made it more convincing that defatted seed, defatted meal and oil solids have similar components and properties and seed meal and crude oil extract are more similar.

4.7.3.2 Quantitation of camelina glucosinolates using two internal standards

To examine the accuracy of the NMR method, known amount (0-2 mg) of glucoraphanin

standard solutions were spiked into *C. sativa* whole seed extracts and the recovery was calculated. The recovery of glucoraphanin ranged from 85.79 to 105.52% (Table 4-9). The low recovery of 85.79% might be caused by several reasons. The first is the human handling and systematic error. The second one is that there may have been some contaminants in the glucoraphanin (≥ 95%) whose protons interfere with the resonance of the compound.

Subsequently the amount of total glucosinolates could be determined by two reference standards: glucoraphanin and DMF. The average glucosinolates level in *C. sativa* whole seed obtained was 20.94 (± 2.14) µg/mL quantified from glucoraphanin, and 20.25 (± 1.23) µg/mL quantified from DMF (Table 4-10). When considering the purity of glucoraphanin (≥ 95%), the measured glucosinolate content in *C. sativa* seed could be estimated to be above 19.89 (± 2.03) µg/mL. While these measured results from the two standards were similar to each other, they were more than 40% higher than the quantitation result obtained when only DMF was added as an internal standard, which was 14.12 (± 0.61) µg/mg, as demonstrated in section 4.7.3.1.

Table 4-9 Accuracy of glucoraphanin level within *C. sativa* matrix in NMR quantitation

Nominal amount (mg)	(n=2)	Calculated amount (mg)	Recovery (%)
0	Mean	0.00	/
	SD	0.00	/
0.5	Mean	0.43	85.79
	SD	0.01	1.60
1	Mean	0.99	99.29
	SD	0.00	0.39
1.5	Mean	1.58	105.52
	SD	0.03	2.12
2	Mean	2.11	105.43
	SD	0.10	5.02

Table 4-10 Total glucosinolate in *C. sativa* seed quantified by glucoraphanin and DMF

Glucoraphanin	DMF	Total glucosinolate (μg/mg) in *C. sativa* whole seed		
(mg)	(mg)	(n=2)	Ref. glucoraphanin	Ref. DMF
0	0.025	Mean	/	18.92
		SD	/	1.23
0.5	0.05	Mean	23.74	20.38
		SD	1.70	1.84
1	0.075	Mean	21.19	21.04
		SD	0.69	0.77
1.5	0.1	Mean	20.18	21.28
		SD	0.96	0.58
2	0.125	Mean	18.64	19.64
		SD	0.55	0.36
		Overall mean	20.94	20.25
		SD	2.14	1.23

Based on the results from section 4.7.1, oil content of *C. sativa* whole seed using Goldfisch extraction was determined to be 35.13%, and the content of the defatted portion could therefore be estimated to be 64.87%. With the defatted portion (64.87%) and the glucosinolate measured from defatted seed (28.96 μg/mg, as shown in section 4.7.3.1), total glucosinolates level in *C. sativa* whole seed could be estimated to be 18.79 μg/mg, but in reality the measured value was 14.12 μg/mg, as was demonstrated in section 4.7.3.1 even when the glucosinolates level in crude oil was neglected. Further more, oil content of *C. sativa* pressed meal using Goldfisch extraction was determined to be 15.30%, and the defatted portion could be estimated to be 84.70%. With this defatted portion and the glucosinolate level measured from defatted meal (31.81 μg/mg, as shown in section 4.7.3.1), total glucosinolates level in *C. sativa* seed meal could be estimated to be 26.94 μg/mg, but in reality the measured value was 24.27 μg/mg, as was demonstrated in section 4.7.3.1 even when the glucosinolates level in crude oil was neglected. In brief, glucosinolate levels in defatted seed materials were higher than other fractions with oil, and the more oil a fraction contained, the fewer glucosinolates were obtained than expected. It was suspected from the series of experiments that the occurrence of oil in the

seed matrix would affect the content of glucosinolates, due to some biological reactions that were induced during general extraction, which remain to be tested. In the current experiment, relatively higher level of glucoraphanin was added to the whole seed extract where oil still existed. As the oxidation of glucoraphanin has been mentioned before (Cataldi *et al*., 2007), the mixing might have caused the reaction between the compound and oil, while the previously underestimated *C. sativa* glucosinolates would be able to preserve.

5. SUMMARY AND CONCLUSIONS

Brassica carinata and *Camelina sativa* are two emerging oilseed crops that are being adapted for cultivation in western Canada. Knowledge of glucosinolates accumulation in oilseed is an important aspect of oilseed value and process development for adding value to these plants. The present project investigated the distribution of glucosinolates during pressing or dehulling of *B. carinata* and *C. sativa* seed. Systematic methods of extraction, detection and quantitation were developed and validated to measure the level of individual glucosinolates in ripe seed of these two species, and these methods can be applied for the measurement in related materials for high throughput analysis.

The major glucosinolate accumulated in *B. carinata* seed is sinigrin. The highly polar nature of this compound required that specific modifications be made for its measurement in a reversed phase HPLC system. Method development was conducted to achieve appropriate retention of the compound sinigrin, in which variables of the ion-pair reagents, buffer strength, buffer pH and organic modifier as well as column temperature and flow rate were studied. Retention factor was the key response to be assessed while the overall chromatographic behavior were also counted in terms of parameters such as mobile phase void time and the number of theoretical plates. The composition of HPLC mobile phase was determined at 100% aqueous tetramethylammonium bromide (TMAB) buffer (10 mM, pH 5) delivered at a flow rate of 1 mL/min. Two C18 columns chosen for comparison were both able to separate the compound in a reproducible manner with appropriate retention, while the Inertsil® ODS-4 C18 column presented higher column efficiency than the Agilent Eclipse C18 column. The method also established good linearity ($R^2 > 0.999$), accuracy, as well as precision through partial validation according to the ICH guidelines (ICH, 2005). These developed chromatographic conditions were later applied to measure the level of sinigrin in *B. carinata* seed, in which 70% (v/v) aq. methanol proved to be a stronger solvent for extracting sinigrin from *B. carinata* seed. The protocol allowed for a corrected estimation of sinigrin concentration in *B. carinata* seed material without the need to conduct the time-consuming desulfation that might cause further calibration problems. Being able to achieve sufficient retention of the polar compound, interference from other less-retained components was largely minimized, and thus assisted in a more accurate estimation of the compound's concentration in real samples. Further study revealed an average

104.4% of *B. carinata* seed matrix effect on the analytical procedure, suggesting an overestimation of the amount of sinigrin. In pressing two lines of *B. carinata* seed, a linear regression of oil yield by pressing and oil content was observed with a high correlation (R^2 > 0.995). It is possible that oil pressing would be sufficiently accurate to estimate seed oil content.

In *C. sativa*, there are three glucosinolates whose structures are similar to glucoraphanin, a glucosinolate that is abundant in broccoli. Detection of these three glucosinolates was first realized in the cold-pressed *C. sativa* oil using HPLC-ESI-MS. Even higher concentrations of the compounds were later found in the processed oil solids. Fragmentation of the compound ions using a developed MRM scanning analysis provided positive confirmation of the occurrence of glucosinolates in *C. sativa* seed material. During this study, it was found that *C. sativa* glucosinolates had weak chromophores and were not readily detected with a UV detector. On the other hand, the fact that these three compounds only differ in the length of the n-alkyl side chain and the similar fragmentation patterns of these glucosinolates suggested that these compounds would be likely to have similar reactions when in an ESI interface. Estimation of the relative proportion of these compounds in a given sample was achieved by use of the extracted ion chromatograms. In addition, quantitation of total glucosinolates was achieved using NMR spectrometry. While authentic standards of these three *C. sativa* glucosinolates were not available, NMR analysis of the glucoraphanin standard revealed the assignment of the S-methyl ($S\text{-}CH_3$) and S-methylene ($S\text{-}CH_2$) protons in the glucosinolate as well as glucose protons attached to the compound. The following ^{1}H NMR, ^{13}C NMR as well as their coupling 2D NMR analysis not only identified the *C. sativa* glucosinolates in the plant extract, but also determined a resonance with a relatively strong signal that was suitable for quantitation. During processing of the *C. sativa* seed, a dehulling treatment involving grinding and aspiration was able to remove significant portion of hull from the seed, which resulted in an increase of both oil content and protein content. It was also noted that there was a significant amount of protein present in the seed hull.

In conclusion, improved chromatography, MS and NMR methodologies have been developed to assess the level of glucosinolates in seed fractions of *Brassica carinata* and *Camelina sativa*. *B. carinata* whole seed contains 29.1 (± 0.6) μg/mg sinigrin, while *C. sativa* whole seed contains 14.1 (± 0.6) μg/mg total glucosinolates; in seed meal recovered from oil extraction, the sinigrin level was 46.7 (± 0.9) μg/mg in *B. carinata*, while the total glucosinolate

level was 24.3 (± 1.2) μg/mg in *C. sativa*. The oil content was 39.5% (± 1) in whole seed of the yellow-seeded *B. carinata*, and 35.1% (± 0.3) in *C. sativa*. When the crude oil from pressing was extracted for glucosinolates, sinigrin level at 0.075 (± 0.005) μg/mg was found in *B. carinata* while the total glucosinolates was shown to make up 7.6 (± 1.5) μg/g in *C. sativa* crude oil.

Exploration of the properties of glucosinolate and their hydrolysis products has been an ongoing area for decades. The ultimate goal is to reveal the full picture of the concentration and presence of these compounds. This work developed systematic methods of extraction, detection as well as quantitation for glucosinolates identified in two oilseed species that are being adapted in western Canada. It increased our knowledge on the distribution of these compounds during primary processing as well as oilseed dehulling. While the ripe seed has been agreed by many to be the great source for glucosinolate isolation, this study further determined that oil solids present as a by-product from primary pressing are an ideal candidate for chromatographic isolation of glucosinolates. This work also demonstrated quantitation of glucosinolates using different analytical techniques, as well as the corresponding extraction processing. Such studies can eventually lead to processes that aim to benefit human and animal health.

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APPENDIX A

LIST OF CHEMICALS

Purchased from Sigma–Aldrich (St. Louis, MO, USA)

Acetonitrile	LC-MS grade
Ammonium acetate	BioXtra, ≥98%
Ammonium bicarbonate	ReagentPlus®, ≥99.0%
Ammonium formate	≥99.0%
Ammonium hydroxide	GR ACS grade
Ammonium sulfate	BioXtra, ≥99.0%
Deuterium oxide	99.9%
Formic acid	LC-MS grade
Methanol	LC-MS grade
(−)-Sinigrin hydrate	≥99.0% (TLC)
Tetramethylammonium bromide (TMAB)	98%

Purchased from EMD chemicals Inc. (Gibbstown, NJ, USA)

Acetonitrile	GR ACS grade
Hexane	GR ACS grade
Methanol	GR ACS grade
N,N-Dimethylformamide	SupraSolv®

Purchased from Cayman Chemical Company (Ann Arbor, MI, USA)

Glucoraphanin	≥95%

APPENDIX B

HPLC METHOD DEVELOPMENT FOR ANALYSIS OF SINIGRIN

Table B-1 Retention times (t_R) of KNO_3 (0.1 M) on two columns

Column	%Acetonitrile	t_R[a] (min)	CV[b] (%)
Eclipse C18	0	1.32	0.00
	1	1.32	0.04
	5	1.31	0.04
	10	1.31	0.04
	30	1.38	0.00
	50	1.32	0.04
	60	1.31	0.04
	70	1.32	0.04
	80	1.35	0.15
	90	1.39	0.04
	100	1.51	0.04
ODS-4 C18	0	1.72	0.06
	1	1.71	0.03
	5	1.68	0.03
	10	1.65	0.06
	30	1.49	0.00
	50	1.37	0.07
	60	1.35	0.04
	70	1.43	0.11
	80	1.48	0.00
	90	1.56	0.04
	100	1.74	0.03

[a] Mean values of retention time (t_R) from three consecutive runs of the same sample.

[b] Coefficient of variance = standard deviation × 100%/mean.

Table B-2 Influence of ion-pair reagents on the chromatographic behavior of sinigrin

Ion-pair reagent		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
			Eclipse C18 column			
Water	Mean	1.53	1.62	0.06	0.21	721.89
(control)	CV (%)	0.08	21.32	379.48	39.58	49.49
Ammonium acetate	Mean	1.52	2.78	0.83	0.15	1712.74
(25 mM, pH 6.78)	CV (%)	0.48	0.67	1.47	5.65	10.38
Ammonium acetate[c]	Mean	1.53	3.14	1.05	0.16	2183.48
(50 mM, pH 6.87)	CV (%)	0.07	0.21	0.40	2.58	5.75
Ammonium formate	Mean	1.54	3.18	1.07	0.12	5639.11
(25 mM, pH 6.46)	CV (%)	0.00	0.52	1.01	12.50	28.61
Ammonium formate	Mean	1.52	3.13	1.06	0.15	2100.38
(50 mM, pH 6.43)	CV (%)	0.04	0.34	0.66	7.90	17.17
TMAB	Mean	1.52	3.01	0.98	0.23	720.63
(5 mM pH 6.54)	CV (%)	0.14	1.25	2.52	9.10	19.79
TMAB	Mean	1.54	3.46	1.24	0.14	2977.68
(10 mM pH 6.64)	CV (%)	0.10	0.52	0.93	7.32	13.13
			ODS-4 C18 column			
Water	Mean	1.74	1.16	-0.33	0.12	868.43
(control)	CV (%)	0.23	14.70	-29.76	54.42	75.75
Ammonium acetate	Mean	1.82	3.44	0.89	0.09	8062.28
(25 mM, pH 6.78)	CV (%)	0.56	0.11	0.23	11.85	29.13
Ammonium acetate[c]	Mean	1.84	3.57	0.94	0.10	7629.89
(50 mM, pH 6.87)	CV (%)	0.03	0.22	0.46	5.17	11.26
Ammonium formate	Mean	1.86	3.62	0.94	0.10	8210.58
(25 mM, pH 6.46)	CV (%)	0.00	0.37	0.77	10.61	23.80
Ammonium formate	Mean	1.87	3.70	0.98	0.10	7599.51
(50 mM, pH 6.43)	CV (%)	0.05	0.24	0.48	11.76	27.48
TMAB	Mean	1.86	3.63	0.95	0.11	6508.60
(5 mM pH 6.54)	CV (%)	0.03	0.31	0.63	6.63	12.48
TMAB	Mean	1.87	4.15	1.22	0.12	6183.23
(10 mM pH 6.64)	CV (%)	0.03	0.17	0.30	4.24	8.49

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of retention time (t_R), retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N) from nine consecutive runs from three samples at three concentration levels.

[c] Sinigrin (1 μg/mL) was not detected; results presented were from six data points.

Table B-3 Influence of buffer strength on the chromatographic behavior of sinigrin

TMAB (mM)		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
			Eclipse C18 column			
0	Mean	1.53	1.62	0.06	0.21	445.22
	CV (%)	0.08	21.32	380.87	39.58	49.49
1	Mean	1.52	2.77	0.82	0.13	2517.86
	CV (%)	0.08	1.84	4.06	9.68	15.36
5	Mean	1.52	3.01	0.98	0.23	1004.38
	CV (%)	0.14	1.25	2.52	9.10	19.79
10	Mean	1.54	3.46	1.24	0.14	3595.69
	CV (%)	0.10	0.52	0.93	7.32	13.13
15	Mean	1.58	3.52	1.23	0.13	3936.23
	CV (%)	0.04	1.10	1.99	3.33	4.69
20	Mean	1.59	3.61	1.27	0.13	4355.48
	CV (%)	0.10	0.71	1.27	2.59	6.41
			ODS-4 C18 column			
0	Mean	1.74	1.16	-0.33	0.12	868.43
	CV (%)	0.23	14.70	-29.76	54.42	75.75
1	Mean	1.85	3.66	0.97	0.12	5176.47
	CV (%)	0.03	1.94	3.92	4.17	9.28
5	Mean	1.86	3.63	0.95	0.11	6508.60
	CV (%)	0.03	0.31	0.63	6.63	12.48
10	Mean	1.87	4.15	1.22	0.12	6183.23
	CV (%)	0.03	0.17	0.30	4.24	8.49
15	Mean	1.96	4.22	1.15	0.14	4757.90
	CV (%)	0.06	0.50	0.93	3.65	7.74
20	Mean	2.04	4.40	1.15	0.14	5267.19
	CV (%)	0.10	0.35	0.65	4.93	10.53

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of retention time (t_R), retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N) from nine consecutive runs from three samples at three concentration levels.

Table B-4 Influence of buffer pH and %acetonitrile on the chromatographic behavior of sinigrin (Con't)

pH	%Acetonitrile		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
				Eclipse C18 column			
3	0	Mean	1.52	4.08	1.69	0.22	2127.11
		CV (%)	0.07	0.51	0.81	18.46	30.94
	0.5[c]	Mean	1.53	3.30	1.16	0.19	1707.01
		CV (%)	0.07	0.28	0.52	12.53	25.03
	1[c]	Mean	1.53	3.31	1.17	0.22	1281.39
		CV (%)	0.08	0.19	0.36	3.45	6.62
	5	Mean	1.51	2.07	0.37	0.14	1250.65
		CV (%)	0.04	0.22	0.82	3.20	6.74
	10[c]	Mean	1.49	1.67	0.12	0.12	1243.56
		CV (%)	0.14	0.78	7.47	24.72	46.61
4	0	Mean	2.08	5.13	1.47	0.28	1960.49
		CV (%)	0.21	1.21	2.04	8.53	17.30
	0.5	Mean	2.19	4.53	1.06	0.20	2843.27
		CV (%)	0.46	1.10	2.14	9.18	18.30
	1	Mean	1.58	3.45	1.18	0.28	921.31
		CV (%)	0.11	1.69	3.12	16.37	29.35
	5	Mean	2.62	3.52	0.34	0.12	5181.63
		CV (%)	1.03	1.04	4.09	6.29	12.40
	10 [c]	Mean	1.71	1.76	0.03	0.14	903.92
		CV (%)	8.91	0.17	6.48	11.95	22.76
5	0	Mean	1.52	4.43	1.91	0.17	3848.05
		CV (%)	0.04	0.55	0.84	5.49	11.64
	0.5	Mean	1.54	3.52	1.29	0.13	4057.75
		CV (%)	0.04	0.47	0.83	0.00	0.93
	1	Mean	1.54	3.54	1.30	0.13	4267.27
		CV (%)	0.04	0.58	1.02	3.45	7.47
	5	Mean	1.55	2.19	0.41	0.05	10656.14
		CV (%)	0.06	0.19	0.64	0.00	0.37
	10	Mean	1.53	1.72	0.12	0.11	1355.56
		CV (%)	0.04	0.13	1.16	0.00	0.26
6	0	Mean	1.52	3.93	1.59	0.24	1565.38
		CV (%)	0.14	0.36	0.58	11.28	20.53
	0.5	Mean	1.53	3.21	1.10	0.20	1557.57
		CV (%)	0.04	0.42	0.80	12.55	23.42
	1	Mean	1.53	3.24	1.11	0.19	1609.15
		CV (%)	0.08	0.35	0.66	9.66	17.82
	5	Mean	1.52	2.08	0.36	0.14	1284.20
		CV (%)	0.04	0.29	1.09	3.66	7.25
	10	Mean	1.51	1.67	0.11	0.14	787.22
		CV (%)	0.04	0.25	2.60	0.00	0.51

Table B-4 Influence of buffer pH and %acetonitrile on sinigrin chromatographic behavior

pH	%Acetonitrile		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
				ODS-4 C18 column			
3	0	Mean	2.10	5.87	1.80	0.20	4868.77
		CV (%)	0.05	0.46	0.72	10.96	20.47
	0.5	Mean	2.04	4.43	1.17	0.17	3974.89
		CV (%)	0.03	0.28	0.53	7.35	15.43
	1	Mean	2.04	4.41	1.16	0.17	3704.74
		CV (%)	0.03	0.22	0.40	8.10	15.54
	5	Mean	1.95	2.76	0.42	0.09	5229.47
		CV (%)	0.05	0.08	0.27	0.00	0.16
	10 [c]	Mean	1.89	2.22	0.18	0.11	2310.89
		CV (%)	0.05	0.29	1.95	9.96	20.31
4	0	Mean	2.09	5.77	1.76	0.19	5412.49
		CV (%)	0.06	0.40	0.62	11.84	21.53
	0.5	Mean	2.04	4.37	1.14	0.16	4290.39
		CV (%)	0.06	0.29	0.55	5.28	10.72
	1	Mean	2.04	4.37	1.14	0.16	4416.92
		CV (%)	0.03	0.42	0.79	5.67	10.93
	5	Mean	1.89	2.74	0.45	0.07	9880.68
		CV (%)	0.03	0.08	0.24	8.04	16.32
	10	Mean	1.82	2.19	0.20	0.11	2198.78
		CV (%)	0.00	0.07	0.39	0.00	0.13
5	0	Mean	1.94	5.43	1.80	0.17	6025.76
		CV (%)	1.87	0.36	0.56	14.03	25.14
	0.5	Mean	1.90	4.16	1.19	0.15	4398.12
		CV (%)	0.09	0.50	0.92	2.98	6.78
	1	Mean	1.91	4.20	1.20	0.14	4777.37
		CV (%)	0.14	0.32	0.59	3.49	7.24
	5	Mean	1.84	2.70	0.47	0.05	16137.30
		CV (%)	0.03	0.07	0.21	0.00	0.13
	10	Mean	1.81	2.07	0.15	0.10	2381.93
		CV (%)	0.00	0.13	0.99	0.00	0.26
6	0	Mean	1.90	5.25	1.76	0.18	5115.71
		CV (%)	0.11	0.44	0.69	10.70	20.27
	0.5	Mean	1.87	4.01	1.15	0.14	4362.63
		CV (%)	0.03	0.52	0.98	3.49	7.50
	1	Mean	1.87	4.05	1.16	0.14	4598.26
		CV (%)	0.05	0.35	0.65	5.54	11.31
	5	Mean	1.84	2.67	0.45	0.07	7774.26
		CV (%)	0.03	0.13	0.41	13.64	30.69
	10	Mean	1.81	2.06	0.14	0.11	2036.84
		CV (%)	0.03	0.55	4.42	4.09	8.93

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of nine consecutive runs from three samples of three levels of concentration.

[c] Sinigrin (1 µg/mL) was not measurable; results presented were from six data point.

Table B-5 Influence of column temperature and %acetonitrile on sinigrin chromatographic behavior

Temp (°C)	%Acetonitrile		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
				Eclipse C18			
25	0	Mean	1.53	4.14	1.71	0.22	2114.05
		CV (%)	0.16	0.64	1.01	14.53	32.02
	1	Mean	1.53	3.50	1.28	0.17	2364.93
		CV (%)	0.00	0.34	0.61	8.49	16.31
35	0	Mean	1.48	3.57	1.41	0.20	1770.35
		CV (%)	0.44	0.97	1.65	11.00	23.13
	1	Mean	1.51	3.14	1.08	0.15	2399.20
		CV (%)	0.04	0.34	0.66	7.90	15.53
45	0	Mean	1.43	3.03	1.11	0.19	1445.07
		CV (%)	0.42	1.12	2.12	9.49	20.74
	1	Mean	1.49	2.85	0.91	0.14	2196.57
		CV (%)	0.08	0.70	1.47	3.49	8.00
55	0	Mean	1.39	2.55	0.83	0.17	1299.12
		CV (%)	0.36	1.04	2.29	11.76	26.48
	1	Mean	1.46	2.52	0.73	0.13	2132.80
		CV (%)	0.10	0.64	1.52	10.41	27.58
				ODS-4 C18			
25	0	Mean	1.94	5.38	1.77	0.18	4914.91
		CV (%)	0.08	0.37	0.58	11.54	26.14
	1	Mean	2.02	4.38	1.17	0.16	4379.95
		CV (%)	0.45	0.49	0.91	5.53	11.74
35	0	Mean	1.96	5.17	1.64	0.18	4675.03
		CV (%)	1.24	0.42	0.67	5.89	12.47
	1	Mean	1.93	4.11	1.13	0.14	4516.60
		CV (%)	0.11	0.33	0.61	5.03	10.76
45	0	Mean	1.91	4.64	1.43	0.16	4881.01
		CV (%)	0.00	0.29	0.49	7.62	16.32
	1	Mean	1.89	3.89	1.06	0.14	4591.53
		CV (%)	0.03	0.36	0.70	3.89	8.20
55	0	Mean	1.88	4.31	1.29	0.15	4613.02
		CV (%)	0.11	0.38	0.68	5.77	11.87
	1	Mean	1.87	3.70	0.98	0.13	4763.34
		CV (%)	0.06	0.57	1.16	3.95	8.55

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of retention time (t_R), retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N) from nine consecutive runs from three samples at three concentration levels.

Table B-6 Influence of flow rates on the chromatographic behavior of sinigrin

Flow rate (mL/min)		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
			Eclipse C18 column			
1.0	Mean	1.52	4.49	1.95	0.17	4024.74
	CV (%)	0.00	0.34	0.52	7.93	17.50
1.1	Mean	1.39	4.15	1.98	0.15	4181.45
	CV (%)	0.04	0.30	0.45	8.26	18.82
1.2	Mean	1.27	3.77	1.96	0.16	3168.08
	CV (%)	0.00	0.25	0.38	8.83	19.27
1.3	Mean	1.21	3.50	1.89	0.17	2278.08
	CV (%)	2.44	0.27	0.41	8.98	20.30
1.4	Mean	1.15	3.27	1.85	0.22	1315.85
	CV (%)	0.18	0.27	0.41	13.52	25.33
1.5	Mean	1.07	2.98	1.77	0.23	964.86
	CV (%)	0.19	0.74	1.15	14.45	28.00
			ODS-4 C18 column			
1.0	Mean	2.09	5.68	1.72	0.20	4506.77
	CV (%)	0.03	0.25	0.39	13.20	26.71
1.1	Mean	1.91	5.18	1.71	0.19	4197.74
	CV (%)	0.03	0.49	0.77	14.37	32.82
1.2	Mean	1.75	4.75	1.72	0.19	3731.84
	CV (%)	0.03	0.17	0.27	13.65	30.97
1.3	Mean	1.61	4.36	1.70	0.18	3462.29
	CV (%)	0.00	0.46	0.73	13.58	29.99
1.4	Mean	1.50	4.06	1.71	0.17	3315.04
	CV (%)	0.04	0.18	0.29	13.56	30.48
1.5	Mean	1.40	3.79	1.71	0.16	3074.11
	CV (%)	0.07	0.15	0.24	10.09	22.02

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of retention time (t_R), retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N) from nine consecutive runs from three samples at three concentration levels.

Table B-7 Analysis of variance of different factors for sinigrin retention during HPLC method development (Con't)

Factors	DF	Mean square	P
Influence of an ion-pair reagent (section 4.1.2)			
C18 column (x_1)	1	0.277	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	2	0.029	< 0.0001
Type of ion-pair reagent (x_3)	6	3.595	< 0.0001
$x_1 \times x_2$	2	0.008	< 0.0001
$x_1 \times x_3$	6	0.094	< 0.0001
$x_2 \times x_3$	12	0.028	< 0.0001
$x_1 \times x_2 \times x_3$	11	0.006	< 0.0001
Error	82	0.000	
Total	123		
Influence of buffer strength (section 4.1.3)			
C18 column (x_1)	1	0.177	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	2	0.019	< 0.0001
Buffer strength (mM) (x_4)	5	4.935	< 0.0001
$x_1 \times x_2$	2	0.006	< 0.0001
$x_1 \times x_4$	5	0.142	< 0.0001
$x_2 \times x_4$	10	0.039	< 0.0001
$x_1 \times x_2 \times x_4$	10	0.008	< 0.0001
Error	72	0.000	
Total	108		
Influence of buffer pH and %acetonitrile (section 4.1.4)			
C18 column (x_1)	1	0.177	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	2	0.006	< 0.0001
Buffer pH (x_5)	3	0.189	< 0.0001
%Acetonitrile (x_6)	4	26.958	< 0.0001
$x_1 \times x_2$	2	0.001	< 0.0001
$x_1 \times x_5$	3	0.105	< 0.0001
$x_1 \times x_6$	4	0.053	< 0.0001
$x_2 \times x_5$	6	0.000	< 0.0001
$x_2 \times x_6$	8	0.001	< 0.0001
$x_5 \times x_6$	12	0.028	< 0.0001
$x_1 \times x_2 \times x_5$	6	0.001	< 0.0001
$x_1 \times x_2 \times x_6$	8	0.000	0.003
$x_1 \times x_5 \times x_6$	12	0.020	< 0.0001
$x_2 \times x_5 \times x_6$	23	0.000	0.159
$x_1 \times x_2 \times x_5 \times x_6$	20	0.000	0.106
Error	230	0.000	
Total	345		

Table B-7 Analysis of variance of different factors for sinigrin retention during HPLC method development

Factors	DF	Mean square	P
Influence of column temperature and %acetonitrile (section 4.1.5)			
C18 columns (x_1)	1	1.103	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	2	0.006	< 0.0001
Column temperature (x_7)	3	1.883	< 0.0001
%Acetonitrile (x_6)	1	4.565	< 0.0001
$x_1 \times x_2$	2	0.000	0.021
$x_1 \times x_7$	3	0.233	< 0.0001
$x_1 \times x_6$	1	0.283	< 0.0001
$x_2 \times x_7$	6	0.000	0.679
$x_2 \times x_6$	2	0.000	0.082
$x6 \times x_7$	3	0.163	< 0.0001
$x_1 \times x_2 \times x_7$	6	0.000	0.589
$x_1 \times x_2 \times x_6$	2	0.000	0.046
$x_1 \times x_6 \times x_7$	3	0.000	0.003
$x_2 \times x_6 \times x_7$	6	0.000	0.484
$x_1 \times x_2 \times x_6 \times x_7$	6	0.000	0.964
Error	96	0.000	
Total	144		
Influence of flow rate (section 4.1.6)			
C18 columns (x_1)	1	0.952	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	2	0.002	< 0.0001
Flow rate (mL/min) (x_8)	5	0.031	< 0.0001
$x_1 \times x_2$	2	0.000	0.005
$x_1 \times x_8$	5	0.028	< 0.0001
$x_2 \times x_8$	10	0.000	0.001
$x_1 \times x_2 \times x_8$	10	0.000	0.001
Error	72	0.000	
Total	108		

Table B-8 HPLC variables estimation model on the retention of sinigrin (Con't)

	Predictors	Correlation with k (Pearson)	Coefficients	P	Model R Squared
Influence of ion-pair reagent					
Eclipse C18 column	x_2	0.068	0.001	< 0.0001	0.588
	x_3	0.762	0.141	0.330	
	constant		0.290	< 0.001	
ODS-4 C18 column	x_2	0.022	0.000	0.809	0.529
	x_3	0.727	0.172	< 0.0001	
	constant		0.102	0.314	
Two C18 columns	x_1	-0.105	-0.082	0.125	0.550
	x_2	0.043	0.000	0.432	
	x_3	0.734	0.157	< 0.0001	
	constant		0.319	0.003	
Influence of buffer strength					
Eclipse C18 column	x_2	0.036	0.000	0.695	0.580
	x_4	0.761	0.045	< 0.0001	
	constant		0.537	< 0.0001	
ODS-4 C18 column	x_2	0.012	0.000	0.916	0.393
	x_4	0.626	0.047	< 0.0001	
	constant		0.452	< 0.0001	
Two C18 columns	x_1	-0.082	-0.081	0.252	0.469
	x_2	0.022	0.000	0.758	
	x_4	0.680	0.046	< 0.0001	
	constant		0.616	< 0.0001	
Influence of buffer pH and %Acetonitrile					
Eclipse C18 column	x_2	-0.042	0.000	0.765	0.842
	x_5	-0.014	0.006	0.700	
	x_6	-0.918	-0.145	< 0.0001	
	constant		1.347	< 0.0001	
ODS-4 C18 column	x_2	-0.025	0.000	0.937	0.829
	x_5	-0.033	0.003	0.870	
	x_6	-0.911	-0.140	< 0.0001	
	constant		1.391	< 0.0001	

Table B-8 HPLC variables estimation model on the retention of sinigrin

	Predictors	Correlation with k (Pearson)	Coefficients	P	Model R squared
Influence of buffer pH and %acetonitrile					
Two C18 columns	x_1	0.037	0.048	0.062	0.836
	x_2	-0.034	0.000	0.785	
	x_5	-0.025	0.005	0.694	
	x_6	-0.913	-0.142	< 0.0001	
	constant		1.297	< 0.0001	
Influence of column temperature and %acetonitrile					
Eclipse C18 column	x_2	-0.036	0.000	0.149	0.959
	x_6	-0.438	-0.267	< 0.0001	
	x_7	-0.875	-0.024	< 0.0001	
	constant		2.233	< 0.0001	
ODS-4 C18 column	x_2	-0.027	0.000	0.321	0.952
	x_6	-0.844	-0.445	< 0.0001	
	x_7	-0.489	-0.012	< 0.0001	
	constant		1.999	< 0.0001	
Two C18 columns	x_1	0.293	0.175	< 0.0001	0.884
	x_2	-0.030	0.000	0.299	
	x_6	-0.597	-0.356	< 0.0001	
	x_7	-0.664	-0.018	< 0.0001	
	constant		1.854	< 0.0001	
Influence of mobile phase flow rate					
Eclipse C18 column	x_2	-0.108	0.000	0.089	0.803
	x_8	-0.889	-0.389	< 0.0001	
	constant		2.393	< 0.0001	
ODS-4 C18 column	x_2	-0.330	0.000	0.014	0.148
	x_8	-0.197	-0.012	0.133	
	constant		1.730	< 0.0001	
Two C18 columns	x_1	-0.870	-0.188	< 0.0001	0.860
	x_2	-0.053	0.000	0.153	
	x_8	-0.317	-0.200	< 0.0001	
	constant		2.343	< 0.0001	

Table B-9 Influence of flow rate and matrix on sinigrin using a Chromolith column

Flow rate (mL/min)		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
				Sinigrin prepared in water		
1.0	Mean	1.70	3.06	0.80	0.08	7267.24
	CV (%)	0.03	0.19	0.44	2.36	4.82
1.5	Mean	1.14	2.06	0.81	0.06	6558.92
	CV (%)	0.05	0.11	0.25	3.05	6.40
2.0	Mean	0.86	1.56	0.82	0.05	6052.94
	CV (%)	0.07	0.15	0.33	2.95	6.00
2.5	Mean	0.69	1.25	0.82	0.04	6086.22
	CV (%)	0.08	0.13	0.29	9.14	23.09
3.0	Mean	0.58	1.05	0.83	0.04	4912.05
	CV (%)	0.00	0.19	0.42	5.30	9.70
3.5	Mean	0.50	0.90	0.83	0.03	4430.07
	CV (%)	0.00	0.15	0.33	7.41	13.76
				Sinigrin prepared in HPLC mobile phase		
1.0	Mean	1.70	3.07	0.80	0.09	6948.76
	CV (%)	0.03	0.10	0.22	5.81	11.63
1.5	Mean	1.14	2.07	0.81	0.06	6309.83
	CV (%)	0.05	0.12	0.26	5.22	10.39
2.0	Mean	0.86	1.56	0.82	0.05	5692.92
	CV (%)	0.07	0.21	0.47	7.77	14.21
2.5	Mean	0.69	1.25	0.82	0.04	5395.27
	CV (%)	0.08	0.15	0.33	7.12	13.35
3.0	Mean	0.58	1.05	0.82	0.04	4823.76
	CV (%)	0.00	0.15	0.34	7.56	13.89
3.5	Mean	0.50	0.91	0.83	0.03	4391.65
	CV (%)	0.00	0.23	0.50	8.31	15.23

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of retention time (t_R), retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N) from nine consecutive runs from three samples at three concentration levels.

Table B-10 Analysis of variance of different factors on the retention of sinigrin using a monolithic C18 column

Factors	DF	Mean square	P
Sinigrin conc. (μg/mL) (x_2)	2	0.000	< 0.0001
Flow rate (mL/min) (x_8)	5	0.002	< 0.0001
Matrix (x_9)	1	0.000	0.261
$x_2 \times x_8$	10	0.000	< 0.0001
$x_2 \times x_9$	2	0.000	0.056
$x_8 \times x_9$	5	0.000	0.005
$x_2 \times x_8 \times x_9$	10	0.000	0.02
Error	72	0.000	
Total	108		

APPENDIX C

HPLC METHOD VALIDATION FOR ANALYSIS OF SINIGRIN

Table C-1 Chromatographic behavior of sinigrin in two matrices on both columns

Matrix		t_m[a] (min)	t_R[b] (min)	k[b]	W_h[b]	N[b]
			Eclipse C18			
Water	Mean	1.43	4.05	1.84	0.21	2011.42
	CV (%)	0.04	0.44	0.68	6.69	15.89
Mobile phase	Mean		4.07	1.86	0.24	1580.97
	CV (%)		0.57	0.87	4.26	8.38
			ODS-4 C18			
Water	Mean	1.94	5.41	1.78	0.21	3817.71
	CV (%)	0.13	0.38	0.59	5.74	11.95
Mobile phase	Mean		5.47	1.81	0.22	3394.22
	CV (%)		0.19	0.29	5.06	10.16

[a] Mean values of the mobile phase void time (t_m) from three consecutive runs of the same sample.

[b] Mean values of retention time (t_R), retention factor (k), peak width at half peak height (W_h), and the number of theoretical plates (N) of twenty-one consecutive runs from seven samples.

Table C-2 Analysis of variance of different factors on the retention and detection of sinigrin

Factors	DF	Mean square	P
Influence on retention of sinigrin			
C18 columns (x_1)	1	0.049	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	6	0.001	< 0.0001
Matrix (x_9)	1	0.012	< 0.0001
$x_1 \times x_2$	6	0.000	0.022
$x_1 \times x_9$	1	0.001	< 0.0001
$x_2 \times x_9$	6	0.000	0.698
$x_1 \times x_2 \times x_9$	6	0.000	0.107
Error	56	0.000	
Total	84		
Influence on peak area of sinigrin			
C18 columns (x_1)	1	266028.808	< 0.0001
Sinigrin conc. (µg/mL) (x_2)	6	3223851.636	< 0.0001
Matrix (x_9)	1	46.354	< 0.001
$x_1 \times x_2$	6	36390.987	< 0.0001
$x_1 \times x_9$	1	629.762	< 0.0001
$x_2 \times x_9$	6	294.170	< 0.0001
$x_1 \times x_2 \times x_9$	6	239.437	< 0.0001
Error	56	2.907	
Total	84		

Table C-3 HPLC-DAD peak areas for sinigrin solutions (0.1 to 5 µg/mL)

Sinigrin concentration (µg/mL)	Peak area[a] (mAU×s)	CV (%)
0.10	1.73	8.28
0.20	3.38	6.28
0.40	6.51	7.37
0.80	12.42	4.25
1.25	17.31	1.77
2.50	34.50	1.37
5.00	69.32	1.37

[a] Mean value of peak areas from four days (three runs per day).

Table C-4 DL and QL determined using two methods

Reference		Method	Result (µg/mL)
ICH, 2005	DL	$DL = \frac{3.3\sigma}{S}$	0.02/0.16
	QL	$QL = \frac{10\sigma}{S}$	0.07/0.48
Vial and Jardy, 1999	DL	$Peak\ area = 3s_{y/x}$	0.09
	QL	$Peak\ area = 10s_{y/x}$	0.43

Table C-5 HPLC-DAD peak areas for sinigrin solutions (10 to 1000 µg/mL)

Sinigrin concentration (µg/mL)	Peak area[a] (mAU×s)	CV (%)
10	140.38	1.06
20	283.38	0.41
40	575.98	0.54
80	1162.74	0.48
100	1455.78	0.98
200	2924.13	0.79
400	5873.48	0.56
600	8866.36	0.15
800	11797.31	0.18
1000	14794.34	0.17

[a] Mean value of peak areas from four days (three runs per day).

Table C-6 Accuracy and precision parameters of the developed HPLC method (Con't)

	Nominal conc. (μg/mL)		Calculated conc.[a] (μg/mL)	Recovery (%)	t_R[b] (min)
Repeatability (n=4)					
Day 1	16	Mean	16.60	103.77	5.70
		CV (%)	0.91	0.91	0.03
	50	Mean	49.70	99.40	5.68
		CV (%)	0.09	0.09	0.01
	160	Mean	160.90	100.56	5.63
		CV (%)	0.03	0.03	0.04
	250	Mean	251.95	100.78	5.59
		CV (%)	0.07	0.07	0.03
	500	Mean	502.30	100.46	5.50
		CV (%)	0.17	0.17	0.05
Day 2	16	Mean	17.17	107.30	5.57
		CV (%)	0.71	0.71	0.02
	50	Mean	51.10	102.20	5.55
		CV (%)	0.06	0.06	0.06
	160	Mean	159.82	99.89	5.51
		CV (%)	0.03	0.03	0.06
	250	Mean	250.09	100.04	5.47
		CV (%)	0.07	0.07	0.05
	500	Mean	501.15	100.23	5.38
		CV (%)	0.04	0.04	0.06
Day 3	16	Mean	16.96	105.98	5.43
		CV (%)	1.00	1.00	0.05
	50	Mean	50.36	100.72	5.41
		CV (%)	0.12	0.12	0.01
	160	Mean	159.22	99.51	5.38
		CV (%)	0.04	0.04	0.07
	250	Mean	250.29	100.12	5.36
		CV (%)	0.02	0.02	0.03
	500	Mean	500.60	100.12	5.31
		CV (%)	0.04	0.04	0.04

Table C-6 Accuracy and precision parameters of the developed HPLC method

	Nominal conc. (µg/mL)		Calculated conc.[a] (µg/mL)	Recovery (%)	t_R[b] (min)
Day 4	16	Mean	16.99	106.18	5.37
		CV (%)	0.34	0.34	0.04
	50	Mean	50.30	100.60	5.35
		CV (%)	0.10	0.10	0.04
	160	Mean	159.19	99.49	5.31
		CV (%)	0.02	0.02	0.04
	250	Mean	250.02	100.01	5.29
		CV (%)	0.11	0.11	0.02
	500	Mean	500.37	100.07	5.24
		CV (%)	0.02	0.02	0.04
Intermediate precision (n=16)					
Inter-day	16	Mean	16.93	105.81	5.52
		CV (%)	1.43	1.43	2.41
	50	Mean	50.37	100.73	5.50
		CV (%)	1.02	1.02	2.42
	160	Mean	159.78	99.86	5.46
		CV (%)	0.45	0.45	2.29
	250	Mean	250.59	100.23	5.43
		CV (%)	0.33	0.33	2.19
	500	Mean	501.11	100.22	5.36
		CV (%)	0.17	0.17	1.90

[a] Calculated from the external calibration curve: y=14.795x-21.11, where y refers to the peak area obtained against the injected concentration x (µg/mL).

[b] Mean value of retention times (t_R) from four days (four runs per day).

APPENDIX D

NMR SPECTRUM FOR CHARACTERIZATION OF CAMELINA GLUCOSINOLATES

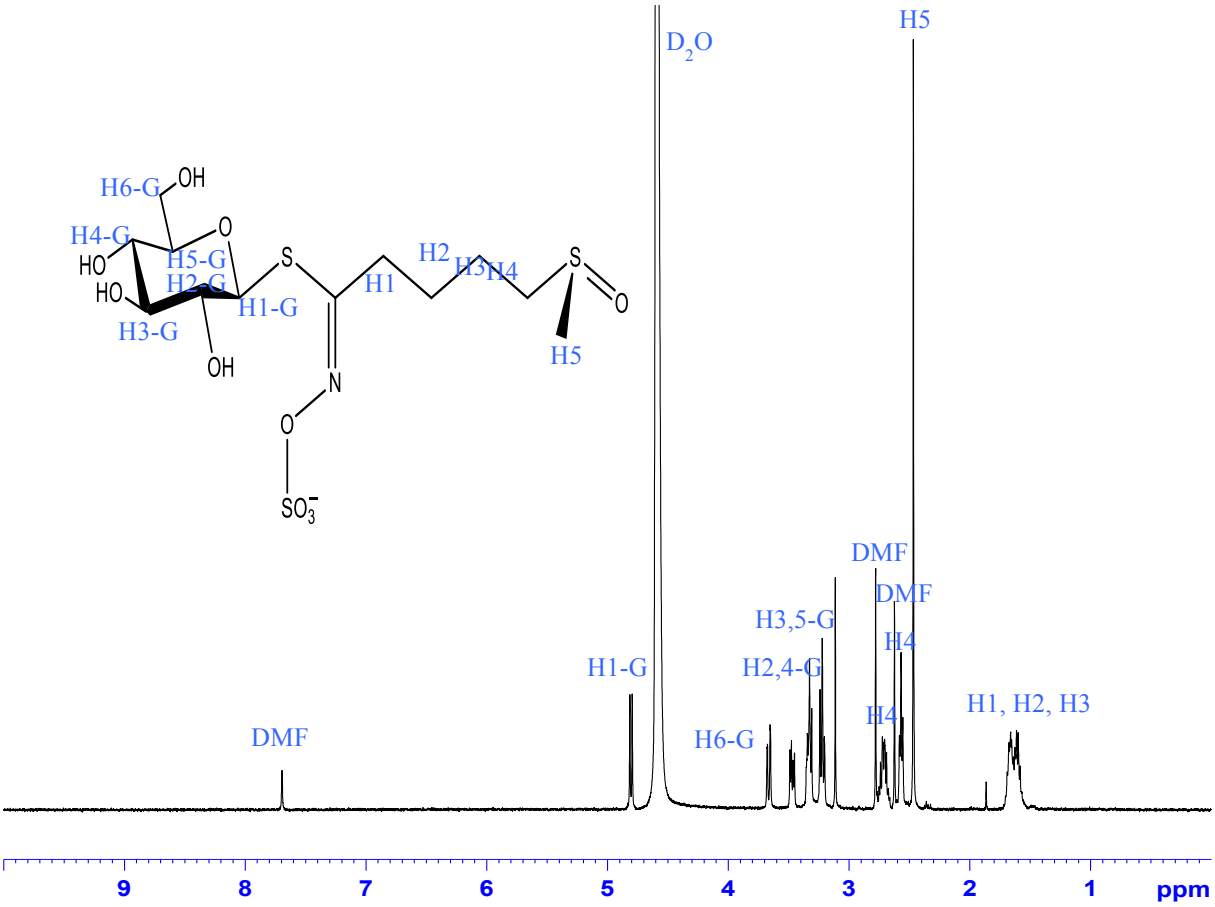

Figure D-1 ^{1}H NMR spectrum of glucoraphanin (1 mg/mL) in DMF solution.

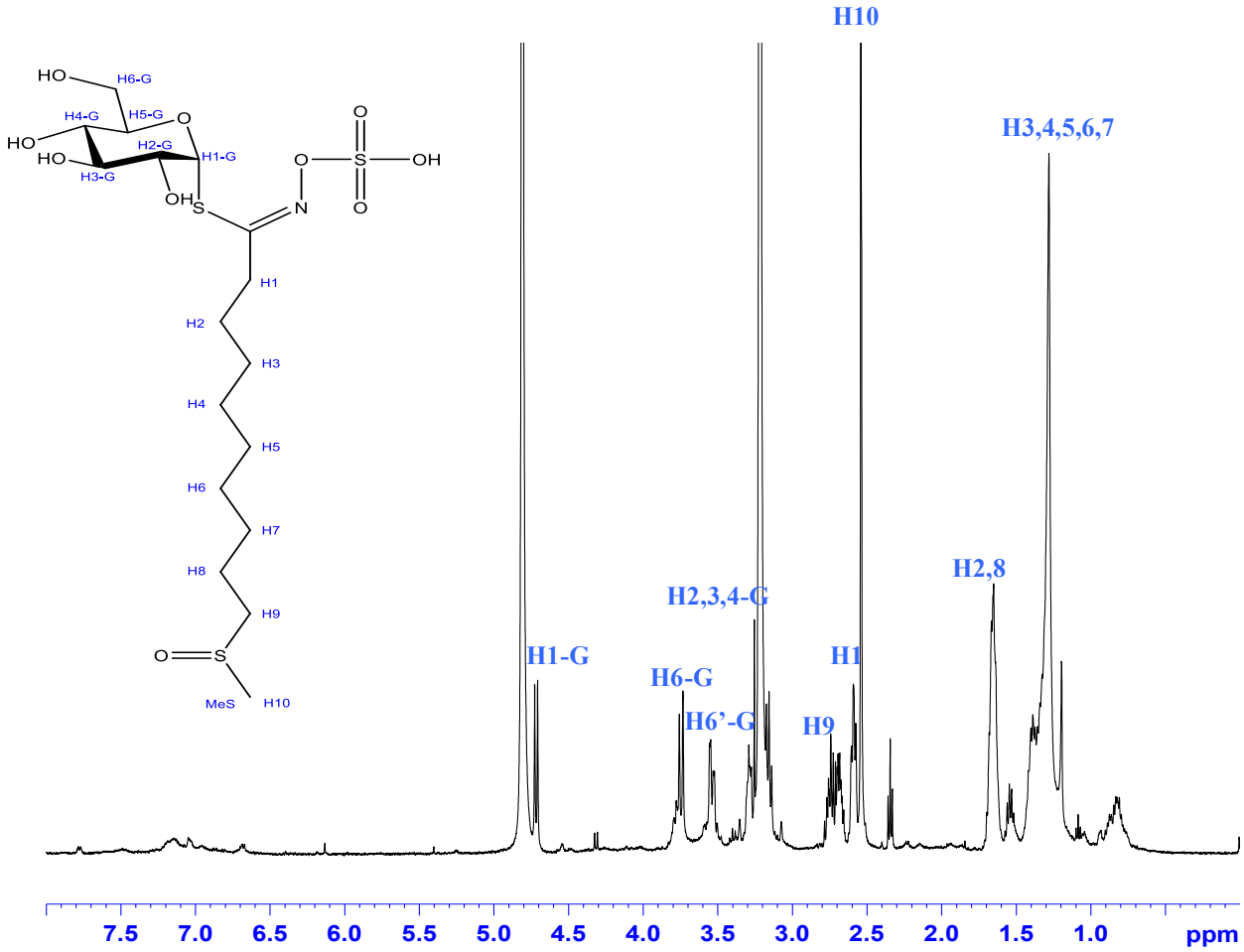

Figure D-2 ^{1}H NMR spectrum of an enriched fraction (in MeOD solution) with m/z 506.

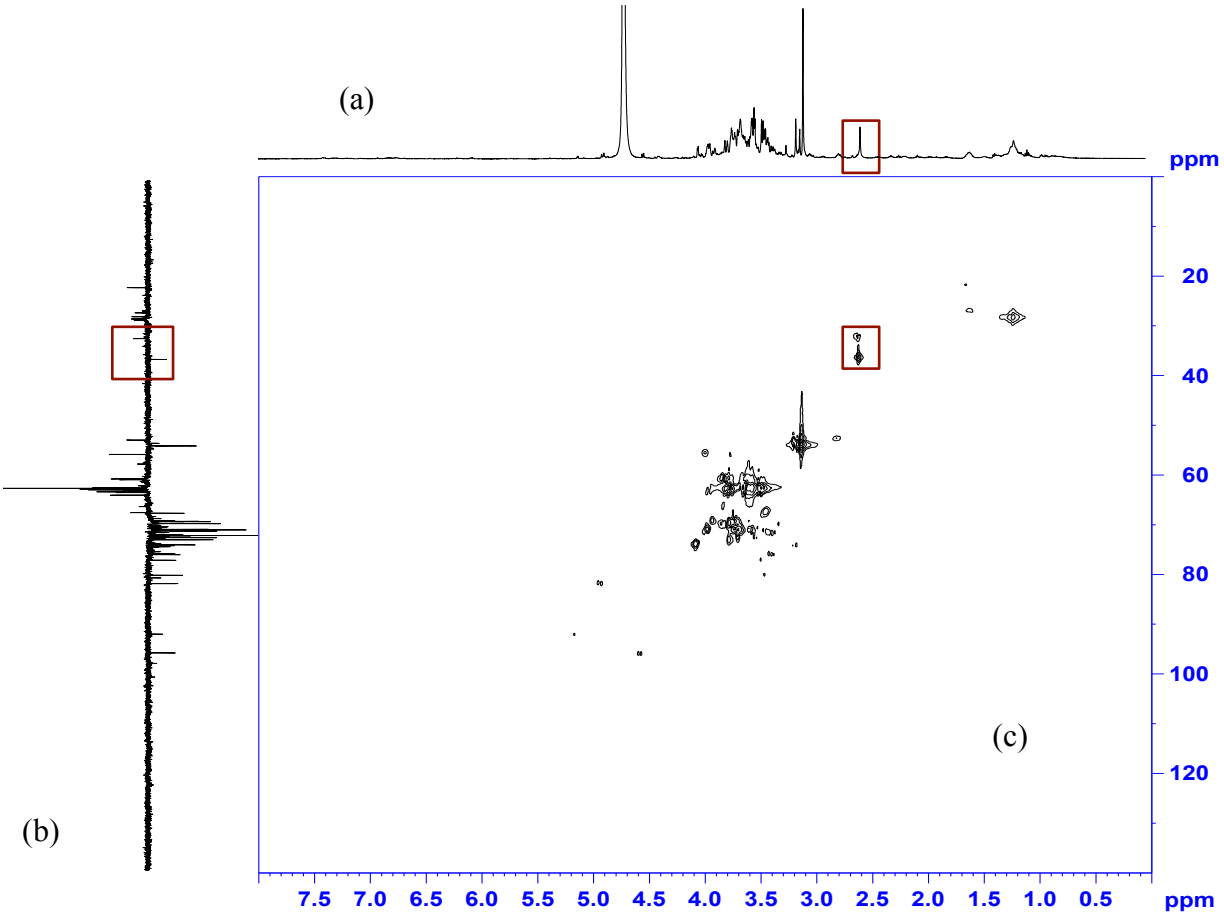

Figure D-3 The ^{1}H and ^{13}C NMR spectra aligned with HMQC (APT) spectrum of *C. sativa* oil solid extract. (a) ^{1}H region; (b) ^{13}C region (apt); and (c) 2D-HMQC region.

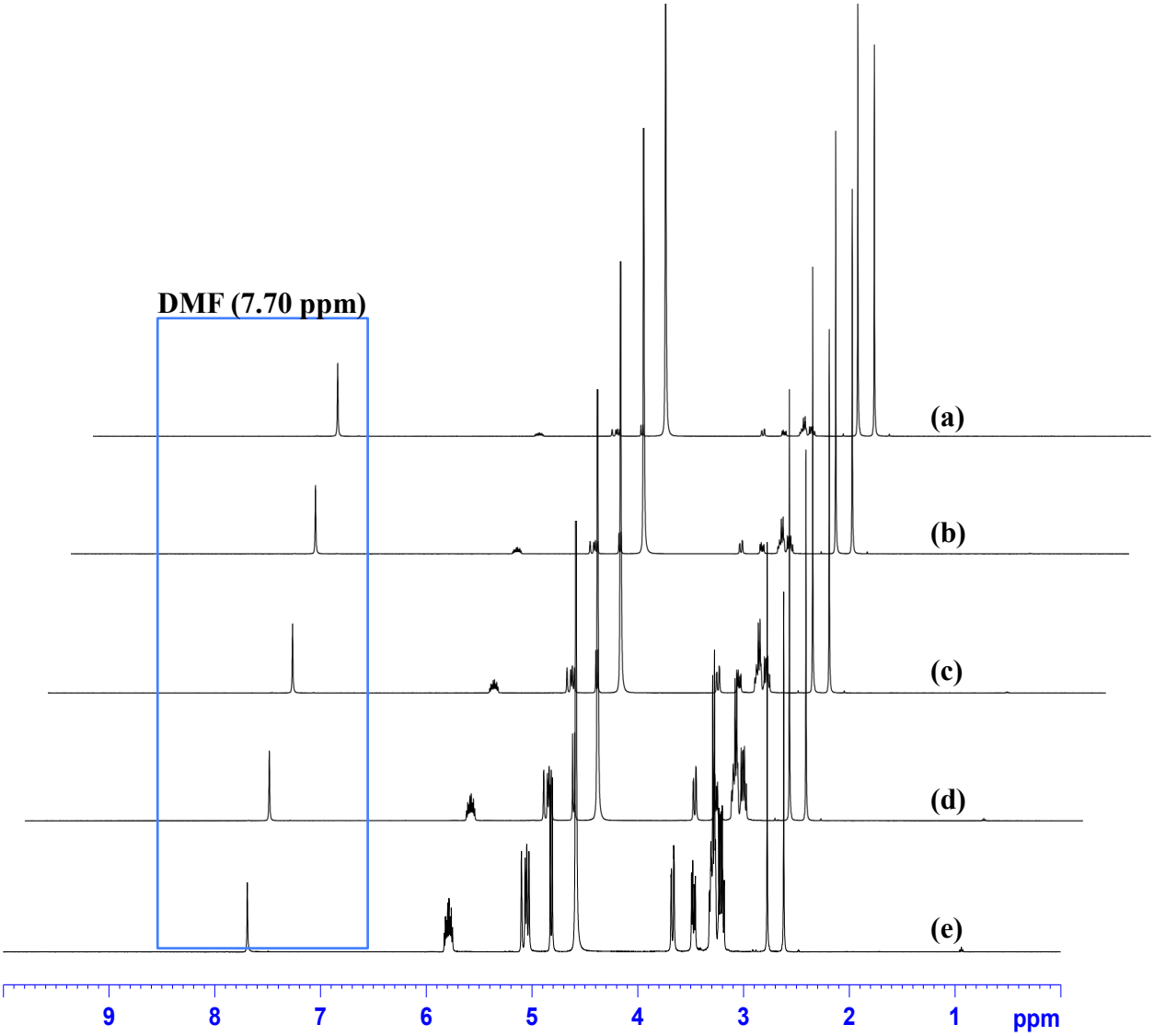

Figure D-4 Overlaid ^{1}H NMR spectra of sinigrin in DMF solution: (a) Sinigrin at 1 mg/mL; (b) 2 mg/mL; (c) 4 mg/mL; (d) 8 mg/mL; and (e) 16 mg/mL.